The evolutionary history of plovers, genus Charadrius
Phylogeography and breeding systems

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Award date:
2014

Awarding institution:
University of Bath

THE EVOLUTIONARY HISTORY OF PLOVERS, GENUS *CHARADRIUS*: PHYLOGEOGRAPHY AND BREEDING SYSTEMS

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

December 2013

Contents

Appendices

Acknowledgments

I owe a massive thanks to everyone who has been involved in my PhD in even the smallest way, for your support, enthusiasm and guidance.

In particular I would like to thank my supervisory team, Tamás Székely, Terry Burke, Pat Lee and Clemens Küpper, whose shared expertise, ideas and positivity at every stage are what made this research possible. Many thanks also to András Kosztolányi, for providing indispensable advice on Part I.

I've been extremely fortunate to spend the past few years in the Molecular Ecology Lab at the University of Sheffield. The supportive, community atmosphere and genuinely great people there have made this PhD an enjoyable "journey". Thanks to: Jenny Armstrong, Steeves Buckland, Debs Dawson, Adam Dobson, Hannah Dugdale, Aliya El Nagar, Lindsay Farrell, Toby Fountain, Pippa Gullett, Jim Hackland, Russell Hall, Gavin Horsburgh, Emily Hounslow, Jamie Hutchison, Kang-Wook Kim, Andy Krupa, Anna Krystalli, Carly Lynsdale, Katy Maher, Maria Elena Maranelli, Filipa Martins, Caitriona McInerny, Cheryl Mills, Nat Oche, Oliver Otti, Celine Pagnier, Karl Phillips, Liam Rasch, Haslina Razali, Anna Santure, Julia Schroeder, Gavin Thomas, Rachel Tucker and Sophie Webster.

I also owe a great deal to the Biodiversity Lab at the University of Bath, and to all those who supported me there during the early stages of my PhD: Araceli Argüelles-Tico, Jack Brown, Kate Ashbrook, Fiona Burns, John Burnside, Martin Hughes, Peter Long, Ross Mounce, Anne O'Connor, Jorge Enrique Parra, James St Clair, Rene van Dijk and Weihao Zhong.

I am thankful for the warm welcome I received as a visitor and to those who assisted with my research at several institutions. Particular thanks to Bernard (Scopus) Amakobe, Sylvester Karimi, Ronald Mulwa and Christine Ochieng at the National Museums of Kenya. Thanks to Mark Adams, Robert Prys-Jones and Douglas Russell of the Natural History Museum at Tring, and also to Eddie Brede and Mike Bruford at Cardiff University.

Last but not least, to my family, Chris, Rod, Dominic and Jonathan dos Remedios, for their unending support,

and to Michael Hutchinson, for always being on my team. Without you, this thesis would be nothing but empty pages.

Summary

Identifying the mechanisms driving the evolution of breeding systems is one of the central goals in behavioural and evolutionary ecology. Understanding the evolutionary history of species of interest is fundamental to this goal. The objectives of this PhD were, firstly, to investigate the role of sex biases in breeding system evolution among closely-related populations and, secondly, to explore the phylogeographic origins of the plovers, genus *Charadrius.* The plovers are characterised by extraordinary ecological and behavioural diversity and are therefore ideal study species for this work. Firstly, I investigated the role of sex biases among offspring in breeding system evolution at the population level across four populations of Kentish and snowy plover exhibiting differing levels of male and female care. Theoretical hypotheses link male-biased offspring sex ratios, mating opportunities and male parental care. I present modest support for this hypothesis: male offspring had higher survival levels than female offspring under particular conditions (Chapter 3), and also a tendency for faster growth (Chapter 4) in two populations with predominantly male parental care in contrast to two populations with more balanced parental care roles. Without a phylogenetic framework for the genus *Charadrius*, evolutionary studies on these diverse shorebirds have been limited in scope. Therefore, secondly, I present the first global, species-level molecular phylogeny of the genus *Charadrius* and outline their phylogeographic origins. I identified the Northern hemisphere (North America, Europe and Siberia) as the origin of the ancestral *Charadrius* species between 19.2 and 36.6 million years ago (Chapter 5). I suggest that early evolutionary diversification occurred due to shifts in the range of ancestral Northern hemisphere species in response to global cooling during the Miocene period, leading to colonisation of the Southern hemisphere (Chapter 5). Thirdly, I present the results of a more in-depth phylogeographic study on the *Charadrius* plovers of Africa. I identified the widespread Kittlitz's plover (*C. pecuarius*) as the closest sister species to the 'critically endangered' St Helena plover (*C. sanctaehelenae*) and 'vulnerable' Madagascar plover (*C. thoracicus;* Chapter 6). Additionally, I identified genetic structure consistent with subspecies status among African populations of the white-fronted plover (*C. marginatus*), three-banded plover (*C. tricollaris*) and chestnut-banded plover (*C. pallidus*), but not the Kittlitz's plover (Chapter 6), with implications for the influence of breeding systems on population-level genetic differentiation. Overall, this thesis both highlights and facilitates several promising new lines of investigation for future research, with the *Charadrius* plovers as ideal study species (Chapter 7).

1 General Introduction

Kentish plover
Artwork by J. dos Remedios

Breeding systems

Breeding systems encompass a highly diverse repertoire of social behaviours carried out by males and females, including courtship, mating and parental care (Reynolds 1996). Efforts to understand breeding system diversity have contributed to driving forward much of the field of behavioural ecology since the 1960s (Wright 1965; Lack 1968; Emlen & Oring 1977; Clutton-Brock 1991; Reynolds 1996; Owens 2006). The roles of the sexes are commonly labelled as either 'conventional' (female-only care and polygyny) or 'reversed' (male-only care and polyandry) based on classical theories that suggested caring females and competitive males to be the most likely evolutionary outcome of anisogamy (large, 'costly' female gametes and small, 'cheap' male gametes; Trivers 1972) and differential rates of reproductive success in relation to number of mates (Bateman principle, Bateman 1948). However, these classical theories have been criticised since their formation for presenting invalid assumptions and over-simplifying the complexities of breeding system evolution (Dawkins & Carlisle 1976; Kokko & Jennions 2008, Klug *et al.* 2010). Contrary to classical expectations, females commonly mate with multiple males and select genetic partners that may differ from their social partner. Even today, the questions of *how* and *why* sex roles and breeding systems have evolved across the animal kingdom remain among the most complex in evolutionary biology.

At the centre of these questions lies the huge diversity observed in parental care behaviour across taxa. For example, male African bullfrogs (*Pyxicephalus adspersus*) dig channels between adjacent pools of water to prevent their tadpoles drying out (Kok *et al.* 1989); female American red squirrels (*Tamiasciurus hudsonicus*) produce a secondary food cache months before conception, to pass to their offspring once they reach independence at the age of ten months (Boutin *et al.* 2000); and female crab spiders (*Diaea ergandros*) display extreme, sacrificial care, providing food and protection for their young over several months before eventually being eaten themselves (Evans 1998). In mammals, 90% of care is provided by females alone (Clutton-Brock 1991); however in birds, biparental care is most common, occurring in 90% of extant species (Kendeigh 1952; Ligon 1999; Bennett & Owens 2002); amphibians exhibit uniparental care which may be carried out by males or females (Summers *et al.* 2006; 2007); among fish, care is largely male-only (Reynolds *et al.* 2002); and among reptiles and invertebrates, any parental care is rare (Zeh & Smith 1985). In addition, there is great diversity in the type of care provided, and the relative effort the male and female parents put into rearing their young (McGraw *et al.* 2010; Smiseth *et al.* 2012).

Parental care, though beneficial for the survival of offspring, is costly to parents in terms of time and energy, reducing their own chances of survival as well as their potential future reproductive output (Clutton-Brock 1991; Balshine *et al.* 2002). It is therefore in the interests of each parent for their partner to take responsibility for the majority of care (Trivers 1972; Westneat and Sargent 1996; Houston *et al.* 2005; Lessells 2012). This difference in the reproductive interests of males and females is a major source of sexual conflict between parents (Parker 1979; Arnqvist and Rowe 2005; Wedell *et al.* 2006). This parental conflict can be resolved in two ways: in species where offspring survival does not require the attendance of both parents, one parent may desert their family, leaving the other to care for the offspring, alternatively parents may cooperate and raise the young together (Houston and Davies 1985; Székely *et al.* 1996; Chapman *et al.* 2003).

Conflict and cooperation have been fundamental ideas in sociobiology since the 19th century (Darwin 1859, 1871; Kropotkin 1902; Wenseelers et al 2010). Both underlie mating behaviour since sexually reproducing species must cooperate to produce offspring despite their often conflicting reproductive interests, and to resolve conflict by avoiding the opposite sex is evolutionary suicide. The theoretical bases of social behaviour include evolutionary game theory, social network theory, interacting phenotypes and social selection. Although sexual conflict has been a subject of interest for many years (Bateman 1948; Trivers 1972; Parker 1979), only since the 1990s has there been increased focus on studying how sexual conflict acts as a driver of evolutionary change and the impacts of this conflict on breeding systems (Lessells 1999; Tregenza *et al.* 2006). The behaviour of each sex may be constrained by trade-offs between mate choice, parental care, competition and advertisement, therefore breeding systems can be considered "the outcome of a battle among competing interests, with opportunities and constraints set by the environment" (Reynolds 1996).

Today, theoreticians and empiricists are still attempting to identify the constraints and trade-offs involved in sex role evolution. Kokko & Jennions (2008) outlined five important components of sex role evolution: 1) the care needs of the offspring (Wilson 1975; Amat and Masero 2004 KPs; AlRashidi *et al.* 2011); 2) sexual selection (variation in mating success, Queller 1997); 3) multiple mating (expected parentage; Davies 1992; Dixon *et al.* 1994); 4) the operational sex ratio (OSR; the ratio of sexually active males to fertilisable females); and 5) the adult sex ratio (ASR; the ratio of males to females in the adult population). Though theoreticians have now identified several potential mechanisms that may drive the evolution of breeding systems,

empirical research is needed to determine the importance of these mechanisms in wild populations.

ASR bias is emerging as one of the key explanatory factors in breeding system variation. Though formerly an area of great conjecture, with the rarer sex thought to have the upper hand in driving mating behaviour (Mayr 1939; Bessa-Gomes *et al.* 2004; Donald 2007), evidence has recently been accumulating that biased ASRs can predict sex roles, mating systems and parental care (Liker *et al.* 2013). To date, very little empirical research has explored the influence of sex ratios on breeding systems across multiple populations of closely related species. It is therefore in this area that I focussed in Part I of this PhD.

Sex ratios and breeding system evolution

After Fisher (1930) outlined his theory on frequency dependent parental investment in sons and daughters and the evolution of sex ratio equality, research interest in unequal sex ratios was minimal. However despite this, reports of skewed sex ratios steadily built up and in 1939, Mayr wrote of the prevalence of sex ratio bias across taxa. Today, our knowledge on the existence of sex ratio bias has increased – in general, birds present a tendency towards male bias whereas female bias is more common among mammals (Donald 2007). Biases may develop at any time in the life history of the individuals in a population: among embryos (primary sex ratio), juveniles (secondary sex ratio), adults (tertiary or adult sex ratio; ASR) or among breeding, sexually active individuals (operational sex ratio; OSR). ASRs differ from OSRs as the former are driven largely by demographic processes, such as mortality, immigration and emigration, whereas the latter are driven by behavioural processes, such as the amount of 'time in' and 'time out' of breeding condition spent by males and females (Székely *et al.* in prep.) However, our knowledge on how sex ratio biases develop in wild populations and how these biases relate to social behaviour is still lacking.

Theoreticians have been debating the link between sex ratios and breeding systems for the last 40 years. The importance of mating opportunities in relation to the spatial distribution of mates and the OSR was emphasised by researchers in the 1970s and 1980s (Emlen & Oring 1977; Breitwisch 1989). However, the idea that ASRs may influence the evolution of breeding systems remained outside mainstream theory until the 1990s, when Queller (1997) suggested that the future success of deserting parents will depend on the ASR, such that male-biased ASRs may bias the mating opportunities towards females, leading to increased female desertion and the evolution of male

parental care. In other words, in a situation where males and females are equally capable of providing care and the only difference is that males are mate-limited and females aren't, males will be selected to provide more care (Queller 1997; Kokko & Jennions 2008). To date, few theoretical or empirical studies have investigated this connection (Grafen & Sibley 1978; McNamara *et al.* 2000, Houston & McNamara 2002, 2005; Kokko & Jennions 2008) but despite initial disinterest from the scientific community, evidence for the importance of ASRs in sex role evolution has gradually been building up (Davies 1992; Jiggins *et al.* 2000; Kvarnemo *et al.* 2007; Liker *et al.* 2013), including evidence from a polyandrous population of Kentish plovers (*C. alexandrinus*) with a male-biased offspring sex ratio and extreme male-biased adult sex ratio (Székely *et al.* 2004; Kosztolányi *et al.* 2011).

Biased ASRs commonly develop at the juvenile stage across a wide range of species (Cooch *et al.* 1997; Oddie 2000; Székely *et al.* 2004; Donald 2007, Hirst *et al.* 2010; Székely *et al.* in prep) therefore studies of offspring sex ratios have the potential to further our understanding of how sex ratios develop as well as their connection to sex role evolution. Though great advances have recently been made in producing theoretical models of the factors influencing the evolution of breeding systems, better integration is required with empirical research to test the value of these models in real-world scenarios.

The small plovers, genus *Charadrius*

Since Darwin (1871) first outlined his theory of sexual selection, shorebirds (order *Charadriiformes*; approx. 350 species) have been used as model organisms in studies of sex roles and breeding systems. Darwin noted reversal of the 'conventional' sex roles among shorebird species including phalaropes (*Phalaropus* spp.), dotterels (*Eudromias morinellus*) and painted snipes (*Rostratula benghalensis*). Breeding systems among shorebirds are highly diverse, ranging from polygyny with intense male-male competition and female-only parental care, to monogamy with equal roles for the sexes in both competition and parental care, to polyandry with sex-role reversal and male-only parental care (Thomas *et al.* 2007). They are likely to express a wide continuum of sexual conflict over both care and mating strategies, making them ideal for investigations into the role of sexual conflict in the evolution of breeding systems (Székely *et al.* 2007).

The small plovers, genus *Charadrius*, are an exemplary group of shorebirds for studies of breeding system evolution due to the exceptionally high diversity exhibited in mating and parental behaviours at the population level, including monogamy, polyandry

and polygyny with biparental, male-only or female-only care (Owens *et al.* 1995; Blomqvist *et al.* 2002; Thomas *et al.* 2007). They have an extremely wide breeding distribution across all continents except Antarctica (Piersma and Wiersma 1996) including subarctic tundra, mountain habitats, temperate grasslands, marine and inland coasts, deserts, semi-deserts and salt marshes. Additionally, many species carry out remarkable long-distance migrations, from breeding grounds in locations including the Arctic Circle to wintering grounds in Africa or South America (van de Kam *et al.* 2004).

Around four times as many shorebird populations are declining than increasing (Stroud *et al.* 2004), largely due to the destruction of wetland habitats at both breeding and wintering grounds (Fernandez & Lank 2008). Unfortunately, the plovers are no exception and although some species are cosmopolitan, distributed widely across multiple continents (e.g. Kentish plover, *Charadrius alexandrinus*), others are 'critically endangered' and confined to small geographic regions (e.g. St Helena plover, *C. sanctaehelenae*; approx. 250 individuals worldwide; IUCN 2013). It is possible that flexibility (or inflexibility) in species' breeding systems may partly explain this global decline. Phylogenetic studies driven by evolutionary questions may therefore have the power to advance conservation for the benefit of these species (Székely *et al.* 2006).

The life history traits of plovers are in some ways very similar to those described for the very first ancestral birds (Wesołowski *et al.* 2004) – they lay their eggs in nest scrapes on the ground and their young are precocial, capable of feeding for themselves within just a few hours of hatching. Furthermore, research on the ancestral origins of parental care in birds has revealed that from a reptilian state of no parental care, the earliest form of care in birds was most likely provided by males, from which shared incubation and female-only care subsequently arose only (Van Rhijn 1984, 1990; Elzanowski 1985; Handford and Mares 1985; Wesołowski 1994; Ligon 1999; Vehrencamp 2000; Varricchio *et al.* 2008). Male-only parental care and polyandry are exhibited by several modern-day plover populations, and therefore studies on the plovers have the potential to reveal new information on the ancestral origins of parental care and sex roles in birds.

The evolutionary relationships among different suborders of the *Charadriiformes* have been investigated thoroughly (Thomas *et al.* 2004; Baker *et al.* 2007) and the order is estimated to have evolved 80-120 million years ago. However, resolution is often lacking at the species level (Thomas *et al.* 2004). Only by understanding the evolutionary history of the plovers can we hope to understand how their diverse breeding systems evolved. Currently, no full molecular phylogeny for the

genus *Charadrius* exists; the best molecular phylogeny based on multiple molecular markers incorporates 9 of 30 *Charadrius* species (Joseph *et al.* 1999). Without this, the evolutionary origins of the group remains unknown and therefore studies on the evolution of breeding systems, migration strategies and the status of endangered species have been without evolutionary context.

Phylogeography

Closely-related species and populations in various locations may exhibit differing mating systems and parental care strategies. To describe and understand the bases of these differences, it is necessary to infer the phylogenetic history of the group within a spatial context. Phylogeography is a discipline that deals with the spatial distribution and evolution of genetic lineages in natural populations. Founded in the 1980s, the aim of this discipline is to determine the historical and contemporary forces that have produced the current genealogical architecture of populations and closely-related species (Avise 2009; Edwards *et al.* 2012). Since the term was coined in 1987 (Avise *et al.* 1987) the discipline has increased substantially in statistical rigor, largely aided by advances in coalescent theory which enable model-based parameter estimation. Coalescent theory, outlined as a suite of probability models in 1982 (Kingsman 1982), uses the observable divergence in a population to model backwards in time in order to estimate the time since a most recent common ancestor (MRCA), at which all gene genealogies 'coalesce' together.

Statistical phylogeography (coined by Knowles & Maddison, 2002) is pushing the discipline on in the 21st century to assimilate the expectations of coalescent theory and other analytical methods with empirical data in order to test phylogeographic hypotheses and obtain estimates of demographic parameters such as divergence times and migration rates (Knowles & Maddison 2002, Templeton 2004, Knowles 2008). Modern approaches do not view the gene genealogy as the central point of analyses but rather a transition variable for connecting data to demographic parameters under a coalescent model (Hey and Machado 2003).

As statistical tools have been advancing, so too have molecular techniques. The emergence of the phylogeographic approach in the 1980s coincided with the introduction of the polymerase chain reacion (PCR) for the amplification of DNA in vitro (Mullis *et al.* 1986; Mullis and Faloona 1987). This was followed by the publication of "universal" PCR primers, applicable across many species, targeting mitochondrial DNA (e.g. cytochrome b, ribosomal genes, control region; Esposti *et al.* 1993; Meyer 1994;

Simon *et al.* 1994; Taberlet 1996). As a result, early phylogeographic studies focussed solely on mtDNA. However, despite past support for the use of mtDNA (see Zink & Barrowclough 2008), with modern advances in techniques for analysis of the nuclear genome, it is now generally accepted that it is best to include multiple nuclear as well as mitochondrial loci for the most robust phylogeographic estimates (Edwards and Beerli 2000, Felsenstein 2006; Carling and Brumfield 2007). Mitochondrial DNA represents only a single locus, and conveys information on matrilineal history only which, though often of interest, may differ from the evolutionary history of the population or species as a whole. Furthermore, the utility of individual genes for investigating the evolutionary diversification of a group, over more ancient or more recent time scales, often differs due to differences in mutation rate. Combining data from multiple genes (into a single 'species tree', Corl & Ellegren 2013) can therefore provide the best estimate of the true evolutionary history of a group.

The discipline of phylogeography is evolving at a rapid pace in line with methodological developments and can be used to address major questions in evolutionary biology. For example, comparative phylogeography can inform conservation priorities, with key conservation areas defined by species richness, endemism and phylogenetic diversity (Brooks *et al.* 2002; Spathelf and Waite 2007); it can be used to investigate the influence of paleogeological and paleoclimatological changes in shaping species distributions (Carstens *et al.* 2005; Alsos *et al.* 2007); and to predict future range shifts that may arise from changes in global climate (Hewitt 1996, 2000) as well as predicting how regional biodiversity may be influenced genetically and demographically (Taberlet and Chedadi 2002; Williams *et al.* 2007).

So far, only a small fraction of the Earth's biodiversity has been studied from a phylogeographic perspective. The pieces of the tree of life are gradually being linked together, however, despite near complete coverage on a large scale (e.g. first draft of a 'complete' global bird phylogeny; Jetz *et al.* 2012), finer scale resolution is often lacking. For groups that exhibit traits of particular evolutionary interest, it is particularly important to study their phylogeography in order to determine species-level relationships and to understand how they reached their present situation. Species may be of interest due to their fascinating natural histories, conservation concerns, taxonomic uncertainties or other species-specific biological motivations.

In Part II of this PhD, I investigate the evolutionary history of the genus *Charadrius*. The phylogeographic approach has the potential to reveal new information about these diverse shorebirds, their global origins, how they reached their current

spatial distribution, and why some species are critically endangered, with populations below 300 individuals worldwide whereas others are widespread with ranges across the northern hemisphere. Furthermore in this new era of integrative research, phylogeographic information can be combined with data on the breeding systems of plovers to provide new insights on how breeding systems can evolve across animal taxa.

The socio-phylogeographic approach

Multi-disciplinary advances are opening up vast new possibilities in the study of breeding system evolution. The socio-phylogeographic approach utilises a phylogenetic framework to investigate the evolutionary bases of social behaviour, integrating research on the many social, ecological and biological factors that lead to complex behaviours (Moore *et al.* 2010). Using this holistic framework, it is possible to test the historical constraints and opportunities, set by the life histories of each sex, that may have predisposed taxa towards particular breeding systems in particular social, biological or physical environments (Robinson *et al.* 2008). By comparing closely related species with differing breeding systems within a phylogeographic context, it is possible to test how selection, as a result of the environment, leads to evolutionary change in the breeding systems of natural populations. Furthermore, the substrates of behaviour are often the same across seemingly distinct groups of organisms, from insects to mammals, with overlap in the genetic, neurological and hormonal components that influence behaviours such as parental care. For example, the insulin pathway of wasps is similar to that found in mammals (Wu & Brown 2006). Studying a single organism or group of organisms therefore has the potential to provide insights across the animal kingdom (Rendall & DiFiore 2007).

With the provision of new molecular genetic tools and updated theoretical ideas, it is up to behavioural ecologists to embrace the opportunity to integrate these fields and to test the latest ideas in wild populations in order to further our understanding of the evolutionary origins of social behaviour. Combining microevolutionary details with macroevolutionary information can lead to new advances in our understanding. Microevolution and macroevolution are part of an evolutionary continuum, only viewed separately from our standpoint in time. In order to understand how breeding systems evolve, we must aim to understand both small scale and large-scale processes. Conducting phylogeographic analyses on the genus *Charadrius* will enable research to progress from studies of mating and parental care strategies within a small number of

closely-related species, to studying *how* and *why* these strategies evolved across the entire genus, across other bird species, and across the animal kingdom.

Thesis Overview

During this PhD, I have taken a two-pronged approach to studying the evolutionary history of the plovers, exploring both macroevolutionary and microevolutionary patterns. In **Part I**, I investigate the role of sex biases among offspring in breeding system evolution at the population level, whilst in **Part II**, I analyse the evolutionary history of the genus *Charadrius*. Studies on these diverse shorebirds have the potential to provide valuable insights on the evolution of breeding systems across taxa. By presenting the first molecular phylogeny of the group, my works aim to pave the way for future, more integrative, socio-phylogeographic research in this area.

Part I: Breeding systems

In **Chapter 2**, I review an essential method for research in evolution, ecology and conservation: molecular sex-typing. The recent development of this technique has made sex identification relatively quick and easy, enabling studies on sex differences in sexually monomorphic species and sex differences among offspring.

In **Chapter 3**, I apply molecular sex-typing methods to investigate sex differences in offspring survival across four populations of Kentish and snowy plover exhibiting differing levels of male and female care. This empirical study is the first to our knowledge to address theoretical hypotheses on the link between male-biased offspring sex ratios, mating opportunities and male parental care across multiple wild populations.

Chapter 4 further explores the existence of sex differences among offspring to determine whether sex differences occur in growth across these same four populations of Kentish and snowy plover. This study has the potential to provide insights on how biased sex ratios might develop among plovers, with implications for the evolution of sex role-reversed breeding systems.

Part II: Phylogeography

Chapter 5 presents the first global, species-level molecular phylogeny of the genus *Charadrius*. Here I aim to determine the global origin of the small plovers and the age of the genus. Additionally, I include several non-*Charadrius* species whose relation to the

genus has been debated for many years, in an attempt to determine their evolutionary relationship to the *Charadrius* genus.

Chapter 6 focusses on the African members of the genus in a population-level, phylogeographic study from a biodiversity conservation perspective. Here, I investigate the phylogenetic affinities of the 'critically endangered' St Helena plover (*C. sanctaehelenae*) and 'vulnerable' Madagascar plover (*C. thoracicus*), and explore the conservation status of the two disparate populations of Chestnut-banded plover (*C. pallidus*), a species that has been largely overlooked by the scientific community in the past.

Finally, **Chapter 7** draws together the main research findings of Parts I and II. In this chapter, I consider the relationship between evolutionary history and breeding systems in the evolutionary history of plovers. I highlight areas for further research with a view to stimulating the integration of these disciplines in future research on the evolution of breeding systems.

During the course of my PhD research, I have additionally worked in collaboration with other members of the Biodiversity Lab, Bath, utilising molecular sex-typing in population-level analyses of sex biases and breeding systems among the 'critically endangered' St Helena plover, the 'vulnerable' Madagascar plover and the co-ocurring Malagasy species, the Kittlitz's plover and White-fronted plover (see **Appendices I - II**).

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2 Molecular sex-typing in shorebirds: a review of an essential method for research in evolution, ecology and conservation

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Abstract

Determining the correct sex of individuals is essential both for research in evolutionary ecology and for practical conservation. Recent molecular advances have produced cheap, quick and reliable methods for sexing birds including chicks, juveniles, immatures and adults. Shorebird researchers have not yet fully utilised these advances. Here we provide an overview of work in this area to date with two objectives: (i) to review the major applications of molecular sexing and findings of shorebird research so far, and (ii) to provide an essential guide on how to carry out molecular sexing using current methods whilst avoiding methodological pitfalls. We encourage shorebird researchers to make better use of molecular sex-typing techniques in studies of conservation, migration, foraging ecology and breeding behaviour.

Published, 2010: Wader Study Group Bulletin 117: 109-118.

Author contributions

NdR: literature search, investigation of methodologies, manuscript preparation
PLML: advice on methodologies, manuscript improvement
TS: manuscript improvement
DD: advice on methodologies, manuscript improvement
CK: advice on methodologies, manuscript improvement

Introduction

The great phenotypic diversity of shorebirds makes them ideal model organisms for the study of major themes in behavioural and evolutionary ecology. Research on shorebirds has helped us to understand the mechanisms behind the evolution of foraging strategies, breeding systems and migration patterns (Delany *et al.* 2009; Hayman *et al.* 1988; Székely *et al.* 2004; van de Kam *et al.* 2004). Determining the sex of individuals is of fundamental importance in these fields, with implications not only for shorebird research but also conservation. Sex ratios provide an important demographic parameter in any population and have been a central theme in evolutionary biology for many years (Fisher 1930; Frank 1990; Hardy 2002; Mayr 1939). Recognising whether the sex ratio within a population fits the 1:1 ratio predicted by Fisher (1930), or diverges towards a higher proportion of males or females is central to understanding behavioural strategies. Research into primary, secondary and tertiary sex ratios, among eggs, chicks and adults respectively, can highlight key factors in the life history of shorebirds (Breitwisch 1989; Mayr 1939). These factors can act at particular points, altering the sex ratio between each stage and determining for example, an individual's chance of surviving to adulthood, the number of potential mates available to them, and whether they should spend time and effort caring for chicks or searching for another mate. Some species, including the Spotted Sandpiper *Tringa macularia* (Oring *et al.* 1983), have even been found to vary their mating system depending upon the tertiary sex-ratio of the population and the mating opportunities available to each sex.

For the many shorebird species under threat and declining in number, knowledge of sex and correct sex-ratio determination is imperative for conservation management. For example, if the breeding population of an endangered species with a polygynous mating system is lacking in females, the effective population size will be reduced, and the situation may be more dire than realised (Weston *et al.* 2004). Donald (2007) found sex-ratio skew among globally-threatened bird species to be greater than for non-threatened species. Attempts at conservation must subsequently focus upon combating the causes of this bias, and reintroduction programmes may be able to redress the balance to improve their status (Donald 2007; Fancy *et al.* 2001; Haig *et al.* 1993).

In species with clear sexual dimorphism in body size, plumage or behaviour, the sex of birds can be identified in the field. However, only 44 of the 213 shorebird species are dimorphic in plumage colouration and of these just twelve are dimorphic throughout the year, in both breeding and non-breeding plumage (Hayman *et al.* 1988). Males and

females of a further 102 species differ in average body size when measured in the hand. However, there is often a degree of overlap in these sizes, which prevents such measurements being fully reliable (Hansen *et al.* 2009). One study of the Red Knot, *Calidris canutus,* revealed incorrect sex assignment in over 20% of individuals estimated morphometrically, whereas molecular sexing was 100% reliable, as confirmed by subsequent gonad analysis (Baker *et al.* 1999). Furthermore, for sexually immature juveniles in the first few weeks after hatching, sex identification in the field is nearly always impossible and molecular sex-typing is essential (Baker *et al.* 1999; Dubiec & Zagalska-Neubauer 2006).

Traits for sexing based on morphometry can be subject to environmental variation both spatially and temporally (Zwarts *et al.* 1996). Van de Pol *et al.* (2008) found body mass and bill length to vary substantially over time in the Eurasian Oystercatcher (*Haematopus ostralegus*) both within and between years. Geographically, males and females often have different growth trajectories in different populations. Biometric differences have been reported between shorebirds at coastal versus inland sites and at different estuaries (Heppleston and Kerridge 1970; Zwarts *et al.* 1996). Thus morphometric sex discriminant functions developed in one population may not work in another, and should be applied with caution.

The development of sex-specific genetic markers for wide use in birds began in the 1990s. Before this, methods for sexing monomorphic species included behavioural observation, examination of the gonads and cytological examination based upon observing differences in the morphology of the sex chromosomes (Dubiec & Zagalska-Neubauer 2006). Early molecular methods included DNA hybridisation techniques, Southern blotting to search for sex-linked minisatellites, and PCR-based techniques such as RAPD (Random Ampification of Polymorphic DNA) and AFLP (Amplification Fragment length Polymorphism) to amplify random fragments of DNA before screening for sex-specificity. As these were not based on highly conserved regions of the genome (that remain unchanged across multiple species), for each species or genus new sex-linked loci had to be identified, making this lengthy and arduous (Lessells & Mateman 1998).

Now, these techniques have been replaced by fast, reliable and cost effective molecular techniques based upon amplification by the polymerase chain reaction (PCR) of conserved sex-specific genetic markers that are widely applicable for the majority of birds. These markers have been quickly adopted for sex-ratio studies, and have opened up large areas of research concerning juveniles, most notably sex allocation, brood sex-

ratios and sex-biased chick survival, which are key to understanding processes at later life-history stages (Andersson *et al.* 2003; Durrell 2006; Reneerkens *et al.* 2005; Sandercock *et al.* 2005; Székely *et al.* 2004). Sex differences are also being revealed in adult survival, reproductive success, parental care and timing of migration, with the composition of populations at breeding, wintering and migratory stopover sites often differing dramatically (Dinsmore *et al.* 2002; Remisiewicz & Wennerberg 2006; Sandercock *et al.* 2005, St Clair *et al.* 2010).

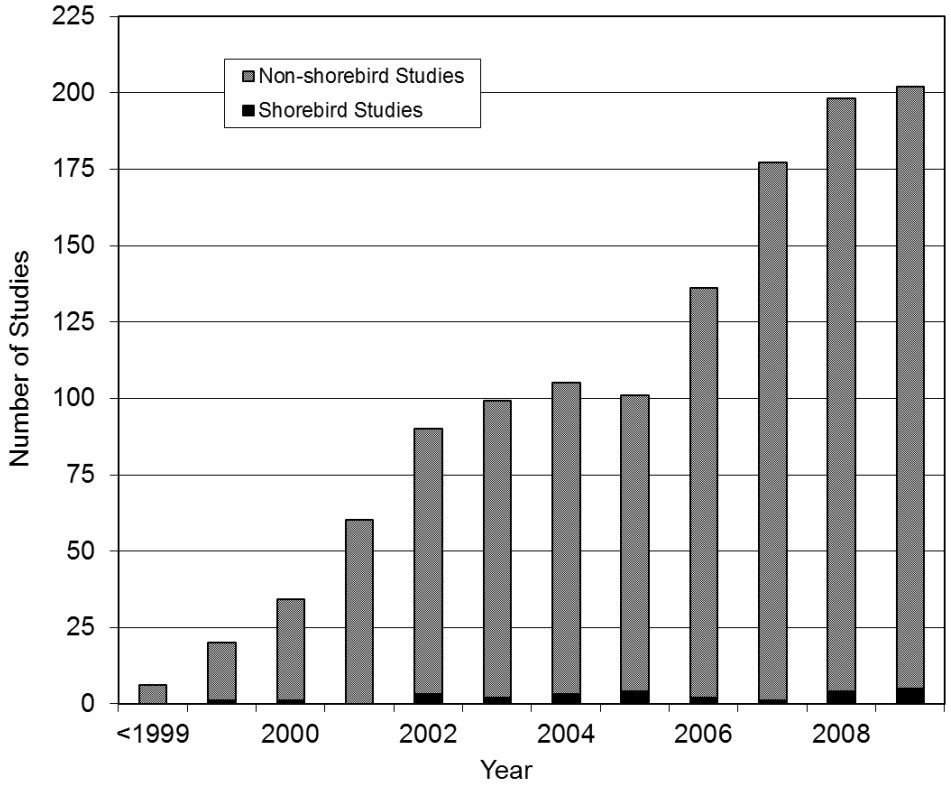
With molecular methods now more accessible than ever before, this review aims to assist shorebird researchers with limited molecular genetic experience by outlining the latest methodologies available for molecular sex-typing and for successfully avoiding pitfalls during the process.

Overview: sex-typing in shorebirds

Molecular sex-typing has seen a rapid rise in popularity over the last decade, currently totalling at least 1,200 published papers in birds alone (ISI Web of Knowledge). Shorebird researchers were slow to capitalise on these new advances and the number of studies utilising molecular sexing has remained relatively low. In a search via the online database ISI Web of Knowledge, we found a year-on-year increase in the number of studies carrying out molecular sexing in birds since the publication of general avian sex-typing markers in 1998, with the exception of 2005. This trend is not replicated for shorebird studies, of which only 27 appear in the last decade, with no more than 5 papers per year (Fig. 1). This is surprising given the enormous research potential these techniques provide for shorebirds.

We identified shorebird studies applying molecular sex-typing via a systematic search in i) ISI Web of Knowledge and Google Scholar (key words: 'molecular sexing* AND shorebirds*', 'molecular sexing* AND *family name*' and 'molecular sexing* AND *common family name*'), ii) citations listed within each publication, and iii) opportunistic findings (Appendix 1). These 40 studies included only 24 of the 213 shorebird species (Hayman *et al.* 1988). Among the species studied, there was a strong bias towards the family *Scolopacidae*, the sandpipers and allies (27 studies on 15 of 88 species), with a reduced focus upon members of the *Charadriidae* family, the plovers and lapwings (nine studies on seven of 65 species). Astonishingly, families including the *Jacanidae, Recurvirostridae, Burhinidae* and *Glariolidae* appear not to have been investigated at all using molecular sexing. This is most likely due to the small number of genotyping studies that have been carried out upon these groups to date.

Figure 1. Studies that utilised avian molecular sex-typing techniques following the publication of universal non-ratite bird sex-typing markers. In total 1,222 studies used molecular sex-typing in birds, of which only 27 were in shorebirds (ISI Web of Knowledge, accessed 13/01/2010).

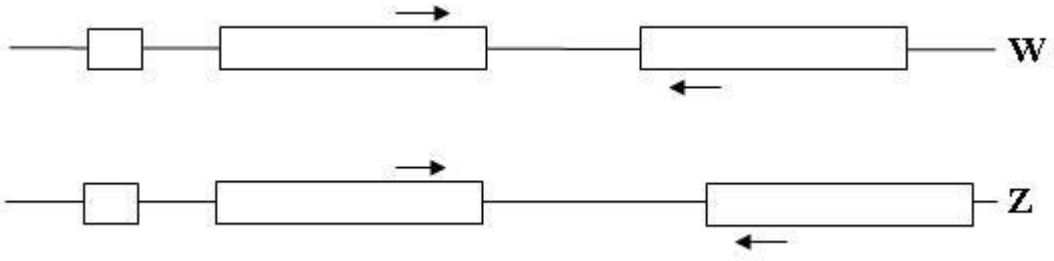


There is also a bias in terms of the topics investigated. Ten of the forty studies used molecular methods in a mechanistic way to verify the reliability of morphological methods for sexing adults in the field. Hypothesis testing was applied in the remaining 30 studies and, of these, six determined juvenile sex ratios in the brood, eight investigated sex-specific traits in breeding and parental care, and eight studied sex differences in migration timings and locations. With so few studies, current knowledge in these areas remains low and each new study reveals novel insights into the life-history strategies of shorebirds.

Methodologies

The majority of molecular markers used in avian sexing identify intrinsic differences in the sex chromosomes, males being the homogametic (ZZ) and females the heterogametic (ZW) sex. The first discoveries of W-linked, female-specific DNA for use in sex identification were made in the early 1990s (Griffiths & Holland 1990; Griffiths *et al.* 1992, Griffiths & Tiwari 1993, 1995; Quinn *et al.* 1990; Rabenold *et al.* 1991) however, methods were specific to particular species and not for broader use across avian taxa. In 1996, the chromo-helicase-DNA binding (*CHD*) gene was identified as a W-specific gene present in most non-ratite bird species (Ellegren 1996; Griffiths *et al.* 1996). It is this *CHD* gene, and its counterpart on the Z chromosome, that are the focus of current molecular sexing techniques. The *CHD* genes function as transcription regulators at the chromatin level and are termed *CHD-Z* and *CHD-W*. In order to distinguish between Z and W chromosomes, the strategy is to PCR amplify a non-coding intron that is of different sizes on the *CHD-Z* and *CHD-W* genes (Griffiths *et al.* 1998). The *CHD* sexing 'primers' target highly-conserved exonic regions flanking the non-coding intron, making them applicable across a wide range of birds (Fig. 2). Males (ZZ) are homogametic resulting in the detection of just one PCR product whereas females (ZW) are heterogametic and the product will appear as two bands / peaks (Griffiths *et al.* 1998).

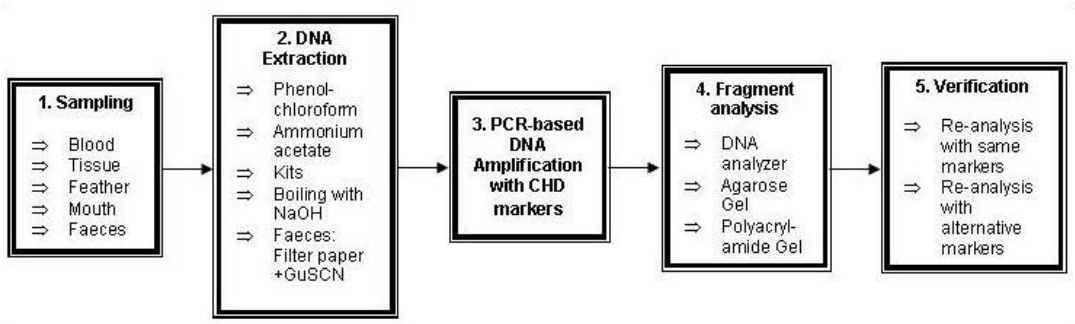
Figure 2. Schematic representation of the W and Z *CHD* regions in birds that are amplified using the *CHD* sex-typing primer sets. Primer binding sites (arrows) are located in highly-conserved exonic regions (boxes) and target introns (lines) of different sizes on the two sex chromosomes.



The sex-typing process

The molecular sex-typing process, from field to lab, can be divided into five key stages (Fig. 3). These consist of i) sampling individuals, ii) extracting DNA from samples, iii) PCR amplification of target regions, iv) fragment analysis and v) verification of results. In recent years various protocols have been developed for each stage. It is therefore important to take note of the differences between and suitability of these methods before starting the molecular sex-typing, in order to work out the best protocol for the species and samples concerned. The following sections outline the most widely used options.

Figure 3. The five stages of the molecular sex-typing process.



I) Sampling methods

The choice of which sampling method to use is constrained by field conditions, training and time. In general, all invasive sampling methods will require specific licences (and often training) which must be obtained from the relevant wildlife and welfare authorities before the sampling begins.

Blood sampling is the most popular and effective method and is recommended whenever possible. Blood is an effective template source for molecular sexing due to the presence of nuclear DNA in the red blood cells of birds. For this, a small (10-100µl) blood sample is taken from the wing or leg veins of birds and collected in capillaries. Evidence from species including shorebirds has repeatedly shown that taking small samples of blood has no detrimental effect (Colwell *et al.* 1988; Sheldon *et al.* 2008). Until DNA can be extracted, blood samples should be stored by one of the following two methods, which each require a different level of preparation:

Buffer: Blood can be stored at 4^{o}C in tubes filled with a buffer such as Queen's Lysis Buffer (0.01M Tris-Cl, 0.01M NaCl, 0.01M EDTA and 1% n-lauroylsarcosine, Seutin *et al.* 1991), dimethyl sulphoxide (DMSO, which is toxic) or at room temperature in absolute ethanol (at a maximum blood:alcohol volume:volume ratio of 1:20). However,

it must be noted that the transport of ethanol on aeroplanes is not permitted for flight safety reasons. When sampling in remote, tropical locations where cold storage is not available, either DMSO or Queen's Lysis Buffer can be used, however, DNA degradation will occur within this time and samples should be transferred to 4^{o}C or below as soon as possible for long-term storage (Kilpatrick 2002).

Filter paper: Alternatively but less commonly, blood from a needle prick can be collected on acid-free filter paper, rapidly dried and kept desiccated as 3mm diameter dried blood spots until analysis (Trudeau *et al.* 2007; Wijnen *et al.* 2008). The reported advantages of this technique, compared to storing the blood in buffer, include faster handling times, with no need for blood collection in capillaries, lower costs and faster DNA extraction (Wijnen *et al.* 2008).

Blood samples collected both in buffer and on filter paper can be stored for several years (Kilpatrick 2002; McNulty *et al.* 2007). However, the quantity (magnitude of 10 to 100 ng/ul) and quality of the DNA obtained from filter paper samples is lower than for samples stored in buffer (Halsall *et al.* 2008). As samples are often used in other analyses it is usually beneficial to have greater magnitude and quality DNA; we therefore strongly recommend storing blood samples in buffer or ethanol.

Tissue sampling is often used when dead embryos, chicks or adults need to be sexed. For recently deceased individuals, common tissues sampled are brain or liver. For museum specimens, footpad samples are commonly used as sources of DNA. During their preparation, museum skins were in the past treated with chemicals such as arsenic. The feet were generally not subject to this treatment and so it is possible to extract higher quality, less degraded DNA from here than from the rest of the body (Mundy *et al.* 1997).

Non-invasive sampling methods are alternatives to blood and tissue sampling in cases where species evade capture, or where there are welfare concerns. For shorebird sex-typing studies three methods may be used, namely the collection of mouth swabs, feathers and faeces.

In ***mouth swab*** sampling, buccal epithelial cells are sampled using a foam-tipped buccal swab or cytology brush which is gently rotated against the inside of the oral cavity and across the tongue of individuals (Handel *et al.* 2006; King *et al.* 2006). The swab can then either be allowed to dry in the air for 10-15 minutes before being placed into a collection tube, or can be suspended immediately in NDS (0.5 M EDTA, 10 mM Tris-HCl, 1%[w/v] sodium lauroyl sarcosine [pH 9.5]). Buccal samples do not yield as much DNA as blood samples (<10ng/µl, Handel *et al.* 2006), nor can they be used to investigate

other measures, for example blood parasites, yet their rapidity makes them a good alternative.

When sampling ***feathers***, it is possible to obtain DNA from plucked or moulted contour feathers, wing feathers or tail feathers. Growing feathers are especially useful due to the presence of dividing cells at their bases and in some cases also blood cells containing DNA (Bush *et al.* 2005). Contour feathers are a less reliable source of DNA, as they depend upon skin cells adhering to the base of the rachis. DNA can be extracted from the basal tip of the calamus (Morin *et al.* 1994) or from the blood clot embedded in superior umbilicus of the shaft, this area having been reported to provide the highest quality DNA from feathers (Horvath *et al.* 2005). The feasibility of using moulted feathers has also been investigated, however it is impossible to assign feathers to individual birds, and results may be confounded by the collection of multiple feathers from the same individual (Bush *et al.* 2005; Mino & Del Lama 2009). In situations where direct contact is not possible, an alternative is the 'feather-trap'. This consists of strips of double-sided adhesive tape, placed close to nests where individuals are likely to pass (Maurer *et al.* 2010). The freshness of feathers collected by this method compared to moulted feathers means that better quality and more DNA can be extracted due to the presence of live cells within them.

Faeces have been widely used as a source of DNA in mammals however few studies have been carried out in birds (Alda *et al.* 2007; Idaghdour *et al.* 2003; Regnaut *et al.* 2006; Robertson *et al.* 1999; Segelbacher & Steinbrück 2001). The quality of the DNA extracted from faeces is lower than that of feathers and various difficulties can occur during PCR as a result (Alda *et al.* 2007). Robertson *et al.* (1999) were the first to successfully utilise avian faecal material to sex-type the kakapo (*Strigops habroptilus*). Regnaut *et al.* (2006) reported up to 98% genotyping reliability based upon amplification of 11 microsatellites in the Capercaillie (*Tetrao urogallus*); although this was only achieved after five labour-intensive repetitions, with initial success at just 69%.

Alternatives to blood and tissue sampling have been gaining popularity in recent years, with the improvement of methods for the extraction of degraded DNA and successful amplification of PCR products. Of the three methods, mouth swab sampling provides the most consistent source of good-quality DNA, followed by feathers and then faeces (Robertson *et al.* 1999; Handel *et al.* 2006; Alda *et al.* 2007). Due to frequent problems with DNA degradation of these samples, we strongly recommend blood sampling as the standard sampling method, yielding the highest quantity of high-quality DNA.

II) DNA Extraction

Genomic DNA extraction is the most crucial step determining the success of molecular sexing. DNA should be extracted from samples such that inhibitors of *Taq* polymerase are not present in the serum (Kramvis *et al.* 1996). Such inhibitors have been found in blood and muscle tissue extracts and can result in failure of PCR amplification (Bélec *et al.* 1998). The standard phenol-chloroform extraction involves incubation of samples with proteinase K in a lysis buffer (e.g. Queen's lysis buffer or SDS based buffers) followed by phenol-chloroform extraction and ethanol precipitation (Maniatis *et al.* 1982). However, this method is laborious, time-consuming and expensive, and unsuitable for large numbers of samples (Kramvis *et al.* 1996). It also relies on the use of chemicals that are toxic to both the environment and humans (Beja-Pereira *et al.* 2009).

Lately, numerous commercial DNA extraction kits have become available. These kits can be applied to a range of different sample types including feathers (Bush *et al.* 2005). They generally take less time to use (~5 hours cf. <24 hours for the standard phenol-chloroform method), are less expensive, and also reduce the time spent handling samples and the risk of contamination.

Ammonium acetate extraction is an alternative that produces high yields of high-quality DNA from bird blood or tissue (Nicholls *et al.* 2000; Richardson et al. 2001). The protocol for this is similar to that of phenol-chloroform extraction, except that ammonium acetate is utilised for the precipitation of unwanted proteins (full protocol available at: http://www.shef.ac.uk/nbaf-s/protocols.html). This method is cheaper to carry out than the commercial extraction kits and furthermore, the ability to adapt it for use in 96-well plates means that high-throughput is possible, with DNA being extracted from up to 192 samples in two days (Whitlock *et al.* 2008).

For avian blood samples an alternative protocol has been developed, reducing the processing time of samples by essentially removing the DNA extraction step (Khatib & Gruembaum 1996). PCR amplification is carried out directly on blood samples, after boiling them with sodium hydroxide to release the DNA from cells (Tomasulo *et al.* 2002), but the yield of high-quality DNA resulting from this method is yet to be established.

For DNA extraction from mouth swabs and feathers, it is possible to use standard commercial DNA extraction kits, phenol-chloroform or ammonium acetate protocols

(Bush *et al.* 2007). For mouth swab samples, prior to each method buccal epithelial cells must be collected in a pellet by centrifuging the samples in sterile water or NDS (Beja-Pereira *et al.* 2009; Meldgaard *et al.* 2004). For feather samples, the base of the rachis should be cut from the rest of the feather.

For faecal samples, an effective technique for DNA extraction has been outlined by Marrero *et al.* (2009) involving the use of fine filter paper soaked in guanidine thiocyanate (GuSCN) to absorb DNA from alimentary tract cells on the exterior surface of faeces.

Once high-quality DNA has been extracted, it needs to be dissolved well to an optimal storage concentration of 100–150 ng/µl by diluting with a low TE buffer or ultra-pure water and should be kept in a freezer (-20°C) until further processing.

III) DNA Quantification and PCR

The optimal amount of DNA for a PCR is 10–50 ng with a concentration of 10–15 ng/µl. Successful PCR can in principle be carried out from just one molecule of DNA, though low concentrations are not recommended as they may result in the amplification failure of single alleles (Kenta *et al.* 2008). If the DNA concentration of the template solution is too high (above ~250 ng), PCR failure will also occur. Common reasons for PCR amplification failure from fresh templates are poor-quality DNA and poorly dissolved stock DNA leading to errors in the amount of DNA introduced to the PCR. DNA concentration can be quantified with a fluorometer, a spectrometer with a DNA quantification program, or less accurately by using gel electrophoresis of a small part of the template on agarose gels in the presence of standards with known concentration. Any RNA present amongst stock DNA will lead to over-estimations of the amount of DNA present. Therefore the RNA concentration within a sample needs to be calculated using the 260:280 nm UV absorbance ratio (Maniatis *et al.* 1982), which can be obtained from the fluorometer / spectrometer reading.

Several molecular markers are available for sexing birds (Fridolfsson & Ellegren 1999; Griffiths *et al.* 1998; Kahn *et al.* 1998). Expected product sizes for the most common primers in a large number of bird species are provided in the BIRD SEX-TYPING database (Dawson *et al.* unpublished data) maintained at the NERC Biomolecular Analysis Facility (NBAF) and available online at: http://www.shef.ac.uk/nbaf-s/birdsexing.html. There are three main sets of primers in general use for all birds, with the exception of the ratites (Table 1): the P2/P8 primers of Griffiths *et al.* (1998); the 1237L/1272H primers of Kahn *et al.* (1998); and the 2550F/2718R primers of

Fridolfsson and Ellegren (1999). The first two sets of primers both amplify the same region, the intron between the CHD helicase and DNA-binding regions on the *CHD* gene. Of the two, the P2/P8 primers are generally preferred as they have proven successful for a greater number of species than the 1237L/1272H primers (Table 1). Kahn's 1237L/1272H primers have additionally been criticised for producing many more non-specific fragments than Griffith's P2/P8 primers (Dubiec & Zagalska-Neubauer 2006). The difference in product size between *CHD-Z* and *CHD-W* is between 10 and 80 bp (P2/P8 data from multiple species extracted from the NBAF BIRD SEX-TYPING database, Dawson *et al.* unpublished data). The 2550F/2718R amplified products of *CHD-Z* and *CHD-W* are between 150 to 250 bp different in size, depending on the species concerned. In favour of the 2550/2718 primers is the comparatively smaller size of *CHD-W* than *CHD-Z* products.

Table 1. Details and number of times cited for three popular *CHD*-linked primer sets used for the molecular sex-typing of non-ratite birds. The number of citations is based on an ISI Web of Knowledge search carried out on 13/01/2010.

Reference	Primer name	Primer sequence (5' - 3')	Size range of products (bp)	Times cited
Griffiths *et al.* (1998)	P2	TCTGCATCGCTAAATCCTTT	350 - 420	825
	P8	CTCCCAAGGATGAGRAAYTG		
Kahn *et al.* (1998)	1237L	GAGAAACTGTGCAAAACAG	300 - 400	85
	1272H	TCCAGAATATCTTCTGCTCC		
Fridolfsson & Ellegren (1999)	2550F	GTTACTGATTCGTCTACGAGA	475 - 700	370
	2718R	ATTGAAATGATCCAGTGCTTG		

Smaller products are more likely to amplify than larger ones in PCR (Toouli *et al.* 2000). Hence with larger *W* and smaller *Z* products (as amplified by primers such as P2/P8), it is possible for *W* fragment amplification failure to go unnoticed due to the presence of the successfully amplified *CHD-Z* product. This results in a greater chance that a female will be mis-sexed as male. However, one disadvantage of the 2550F/2718R primers is that they can be used successfully in fewer species than the P2/P8 primers.

In PCR, target regions of DNA for sexing can be amplified with either one marker at a time, in a singleplex (one reaction per well), or more than one marker at a time, in a multiplex (several reactions per well). Singleplex reactions should be used when the size of products amplified is to be analysed manually on an agarose gel or if the product sizes of markers overlap. Multiplexes can be carried out when fragment analysis is to be

carried out on an automated DNA analyser, or on agarose gel when the sizes of PCR products are known and they are not overlapping.

IV) Fragment Analysis

Analysis of the size of *CHD* products amplified in PCR can be carried out in two ways, either by gel electrophoresis or by automated DNA analysers.

In both processes, DNA fragments will be separated according to size using an agarose, polyacrylamide or polymer matrix. Separation by gel electrophoresis works well when the products differ in size by approximately 35 bp or greater (on 2% agarose gel) and when the products are 0-500 bp in size. However, the resolution is low and it is not possible to determine the precise number of base pairs in each fragment so difficulties will occur if the Z and W products are of similar size, or if *CHD-Z* is polymorphic, in which case heterozygous males could be incorrectly recorded as females (Dawson *et al.* 2001). It must be noted that higher resolution can be attained using gels with a higher agarose concentration (e.g. 2% agarose to 35 bp, 3% agarose to 6 bp) and that acrylamide gels provide higher resolution than agarose.

DNA analysers provide automated fragment analysis and can resolve product sizes to 1-bp accuracy. Hence, where the size of *CHD-Z* and *CHD-W* alleles is known, it is easy to identify Z/W polymorphisms within a population where several known sex control individuals are available for both sexes. To visualize the products, the sexing primers must be fluorescently labelled. Depending on the type of DNA analyser and the number of its capillaries, high throughput is possible (16 to 48 capillaries). Additionally, the products of several sex-typing markers can be analysed in parallel to reduce the possibility of typing errors based on a single marker.

In the past, automated fragment analysis was expensive and not widely available. Today however, the price of automated fragment analysis has dropped significantly and its use is highly recommended to avoid problems that may commonly occur during molecular sex-typing (see section V).

V) Verification of Results

In the assessment of sex-typing marker sets for a particular species, the sex of individuals needs to be initially established through, for example, gonad analysis, clearly dimorphic phenotypes or observed matings. Once the assessment has been finished, it is still important to gain assurance of the results of each sex-typing PCR. This can be done

simply by repeating molecular sexing using several known male and female samples, or by utilising more than one molecular sex-typing marker. The accuracy and reliability of results and ease of sex-typing is greatly affected by the method of analysis used to determine the size of the PCR products. With automated fragment analysis the possibility of error is minimal, particularly when multiple sex-typing markers are analysed; however, when utilising gels for fragment analysis a number of pitfalls have been known to occur.

Pitfalls in molecular sex-typing

Sources of error include firstly allelic dropout, which can cause real females to be assigned as males as a result of the preferential amplification of just the *CHD-Z* allele (Robertson & Gemmel 2006). Allelic dropout occurs more frequently when Z and W products differ in size by 150 bp or more and usually the larger product will drop out (Toouli *et al.* 2000). Secondly, a lack of detectable difference in the size of the *Z* and *W* products can lead to all individuals (including females) being mistakenly assigned as males if no known sex controls are included (Dawson *et al.* 2001). Thirdly, heteroduplex molecules may form when different *CHD-Z* alleles interact during PCR. In shorebirds, Casey *et al.* (2009) highlighted this occurrence in the upland sandpiper (*Bartramia longicauda*), showing that *CHD-Z* products could anneal together to produce aggregations of similar size to the *CHD-W* product. Finally and most commonly reported, inaccuracy may occur as a result of *Z*-polymorphisms in males, such that males may be heterozygous for the *CHD-Z* gene (ZZ') (Dawson *et al.* 2001). The amplification of two differently sized *CHD-Z* products results in the appearance of two bands in electrophoresis, meaning that a male can be confused for a female (Dawson *et al.* 2001). Z-polymorphisms have thus far been reported in approximately 20 bird species (e.g. Casey *et al.* 2009; Dawson *et al.* 2001; Lee *et al.* 2002; Lengyel *et al.* unpublished data; Küpper *et al.* 2009; Schroeder *et al.* 2010). So far polymorphisms have not been detected on the W chromosome *CHD* copy possibly because of its significantly lower mutation rate, nucleotide diversity and genetic variability. It has been suggested that selection may well be acting to streamline this chromosome, maintaining the minimum number of genes and alleles (Montell *et al.* 2001).

Solutions to the pitfalls of molecular sex-typing

Misidentification due to allelic dropout, heteroduplex molecules and Z-polymorphisms can be overcome in three ways. Firstly, utilising more than one sex-typing marker will

increase the reliability of the result. It may be possible to design additional Z- or W-specific primers unique to a study species based upon the sequence amplified using the known *CHD* markers (Bantock *et al.* 2008; Shizuka & Lyon 2008) or an alternative avian sex-linked gene e.g. *SPINZ/SPINW, UBAP2Z/UBAP2W* and *ATP5A1Z/ATP5A1W* (Handley *et al.* 2004). However, for many shorebird species very little sequence data are currently available, therefore, before this can be achieved at least part of the locus of interest must be sequenced in the species concerned.

Secondly, the most common pitfalls of molecular sex-typing can be avoided if a DNA analyser is used. The accuracy of DNA analysers means that Z-polymorphism can be identified. Moreover, heteroduplex molecules are no longer a problem, as PCR products are initially denatured prior to analysis, thus preventing their aggregation (Casey *et al.* 2009).

Thirdly, care must be taken in the collection and storage of samples, whether blood, other tissue or non-invasive samples, in order to ensure that high-quality template DNA is used. Where only low-quality, degraded DNA is available, e.g. ancient, museum specimens with short, sheared DNA, primers can be developed for shorter targets in the *CHD* gene (less than for example 250 bp, Bantock *et al.* 2008).

In general, when choosing molecular markers for sex-typing, one should seek those most appropriate for the study species. If this species has not previously been sexed in this manner, it is advisable to first test the applicability of both the P2/P8 and 2550F/2718R primer pairs (Table 1) using five or more known-sex individuals of each sex. Finally, if it is necessary to design new markers, larger numbers of known-sex birds of each sex must be used for validation.

Conclusions

Molecular sex-typing has enabled avian biologists and conservationists to simply and effectively determine the sex of large numbers of individuals. Shorebird researchers should embrace this established technique to explore evolutionary and ecological trends within and across populations and species. Molecular sexing can help us to understand sex differences and the roles of the sexes at every life-history stage, revealing information about evolutionary events in the past and information that may help us to conserve shorebird species long into the future. In the coming years, studies on shorebirds should endeavour to make far greater use of molecular sex-typing and, in doing so, will undoubtedly deliver important information on these birds' highly diverse strategies of breeding, parental care and migration.

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Appendix 1: Summary of shorebird studies utilising molecular sex-typing. * study not identified via ISI Web of Knowledge.

Species	Marker	Sample Type	Fragment Analysis	Error Rate (%)	Verification	Topic	Outcome	Reference
Actitis hypoleucos	P2/P8; 3007/ 3112; P2/P3	Blood	Automated & 2% Agarose Gel	0	Multiple markers; behaviour	Juvenile sex ratio	Shift in early to late broods from male- to female-biased.	Andersson *et al*. (2003)
Actitis macularia	P2/P8; 3007/ 3112; P2/P3	Blood	Automated & 2% Agarose Gel	0	Multiple markers; behaviour	Juvenile sex ratio	No bias in sex ratio.	Andersson *et al.* (2003)
Arenaria interpres	P2/P8	Blood	2.5% Agarose Gel	Not stated	None	Juvenile sex ratio	Male-biased brood sex ratios.	Reneerkens *et al.* (2005)
Bartramia longicauda	P2/P8; 2550F/ 2718R	Blood	3% Agarose Gel	4.4 (P2/P8 only); 0 (with 2550F/ 2718R)	Two markers; Mated pairs sampled; Restriction enzyme	Methodology test	Heteroduplex molecules can cause misidentification of males as females.	Casey *et al.* (2009)
Calidris alba	P2/P8	Blood	2.5% Agarose Gel	Not stated	None	Juvenile sex ratio	No bias in brood sex ratio.	Reneerkens *et al.* (2005)
Calidris alpina	P2/P8	Blood	3.5% Agarose Gel	Not stated	None	Methodology test	Molecular and phenotypic sexing by morphometrics are both accurate.	Meissner *et al.* (2008)
Calidris alpina	P2/P8	Blood	2.5% Agarose Gel	Not stated	None	Juvenile sex ratio	Male-biased brood sex ratios.	Reneerkens *et al.* (2005)
Calidris canutus	P2/P3	Blood	4% Agarose Gel	0	Gonad analysis	Methodology test	Molecular sexing is more accurate than phenotypic sexing.	Baker *et al.* (1999)
Calidris canutus	P2/P3	Blood	4% Agarose Gel	Not stated	None	Adult survival	No difference in annual survival between the sexes.	Brochard *et al.* (2002)*
Calidris canutus	P2/P3	Blood	4% Agarose Gel	Not stated	None	Migration; Metabolism	Metabolic trade-offs occur in migrants to save energy in resource-limited conditions.	Buehler *et al.* (2009)*
Calidris canutus	P2/P3	Blood	4% Agarose Gel	Not stated	None	Migration	Males skip stopover more often than females.	Nebel *et al.* (2000)

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Calidris canutus	P2/P3	Blood	4% Agarose Gel	Not stated	None	Breeding	Individuals switch from monoester to diester preen wax in courtship and incubation.	Reneerkens *et al.* (2002)
Calidris canutus	P2/P8	Blood	2.5% Agarose Gel	Not stated	None	Juvenile sex ratio	No bias in brood sex ratio.	Reneerkens *et al.* (2005)
Calidris canutus	P2/P3	Blood	4% Agarose Gel	Not stated	None	Moulting; Metabolism	Basal Metabolic Rate increases during moulting, linked to endocrinal changes.	Vézina *et al.* (2009a)*
Calidris canutus	P2/P3	Blood	4% Agarose Gel	Not stated	None	Digestion; Migration	Migrants maintain nutrient stores to buffer against food shortages.	Vézina et al. (2009b)*
Calidris maritima	2550F/ 2718R	Feather	1.5% Agarose Gel	Not stated	None	Migration; Methodology test	Male-biased sex ratio at stopover; phenotypic sexing by bill length has 3% error rate.	Hallgrimsson *et al.* (2008)
Calidris maritima	2550F/ 2718R	Blood	Agarose Gel ($conc^n$ unknown)	Not stated	None	Migration	More males than females are present at stopover in Iceland throughout the season.	Summers *et al.* (2009)*
Calidris mauri	P2/P8	Feather	3% Agarose Gel	Not stated	None	Population sex ratio	Sex bias in the population is not due to sex-biased predation.	Nebel *et al.* (2004)
Calidris temminckii	P2/P8	Blood	2% Agarose Gel	Not stated	None	Breeding	Females are larger and in better condition than males.	Lislevand *et al.* (2009)
Charadrius alexandrinus	P2/P8	Blood	3% Agarose Gel	Not stated	None	Juvenile and adult sex ratio	Male biased sex ratio linked to differential survival of juveniles.	Sandercock *et al.* (2005)
Charadrius alexandrinus	P2/P8	Blood	3% Agarose Gel	0	Repetition; Phenotype comparison	Juvenile sex ratio	Proportion of males increases with brood age.	Székely *et al.* (2004)
Charadrius modestus	P2/P8	Blood	Automated	0	Mated pairs sampled	Parental sex roles	Unusual incubation, but incubation is shared evenly between the sexes.	St Clair *et al.* (2010)*
Charadrius montanus	1237L/ 1272H	Feather	Agarose Gel ($conc^n$ unknown)	15	None	Parental care	Survival rates in male-tended nests exceed female-tended.	Dinsmore et al. (2002)

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Haematopus bachmani	P2/P8	Blood	Automated	0	Second species-specific marker.	Methodology test	Molecular sexing shows phenotypic sexing by eye flecks to be 94% accurate.	Guzzetti *et al.* (2008)
Haematopus ostralegus	P2/P8	Not stated	3% Agarose Gel	Not stated	None	Juvenile sex ratio	Primary sex ratio biased towards the rarer sex in the population.	Durrell (2006)
Haematopus ostralegus	P2/P8	Feather	3% Agarose Gel	6.25	Gonad analysis	Methodology test	Molecular sexing is more accurate than phenotypic sexing.	Watson *et al.* (2004)
Limosa limosa	P2/P8; 2550F/ 2718R	Feather	3% Agarose Gel	0	Two markers; Behaviour	Methodology test	Molecular sexing is accurate and can improve phenotypic sexing techniques.	Gunnarsson *et al.* (2006)
Limosa limosa	2550F/ 2718R	Blood	Not stated	Not stated	None stated	Digestion study	Females show lower faeces energy density and higher assimilation efficiency.	Santiago-Quesada *et al.* (2009)
Limosa limosa	P2/P8; 2550F/ 2718R	Blood	Automated; 3% Agarose Gel	0	Two markers	Methodology test	Molecular sexing is more accurate than phenotypic sexing.	Schroeder *et al.* (2008)
Limosa limosa	P2/P8; 2550F/ 2718R	Blood	Automated	0	Two markers	Fitness & plumage	Less ornamented males are fitter.	Schroeder *et al.* (2009)
Limosa limosa	M5/P8	Blood; Skin	Automated	Not stated	None	Fitness & genotype	Z-Polymorphism in both sexes correlates with pale breeding plumage, high body mass, early breeding and large eggs.	Schroeder *et al.* (2010)*
Limosa limosa	2602F/ 2718R	Blood	Automated	Not stated	None	Microsatellite loci test	Microsatellite loci were not sex linked.	Verkuil *et al.* (2009)*
Lymnocryptes minimus	P2/P8	Blood	3% Agarose Gel	Not stated	None	Methodology test	Males and females differ in 7 of 9 morphometric traits, 99% reliable for sexing.	Sikora *et al.* (2007)
Philomachus pugnax	P2/P3	Blood	4% Agarose Gel	0	Gonad analysis	Breeding	Female mimics identified as 'faeder' males.	Jukema *et al.* (2006)*
Philomachus pugnax	P2/P8	Not stated	Polyacrylamide Gel (conc^{n} unknown)	0	Phenotype comparison.	Juvenile sex ratio	Females in better condition produce more daughters than other females.	Thuman *et al.* (2003)
Philomachus pugnax	P2/P3	Blood	4% Agarose Gel	Not stated	None	Migration	Faeder males winter and migrate with larger lekking males.	Verkuil *et al.* (2008)*

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Scolopax rusticola	P2/P8	Blood	2% Agarose Gel	0	Alternative restriction enzyme; Sequencing.	Methodology test	Molecular sexing is easy, reliable and robust.	Väli *et al.* (2002)
Thinornis rubricollis	P2/P8 + Hae III	Blood	Agarose Gel (conc^{n} unknown)	0	Behaviour; Mated pairs	Adult sex ratio	Male-biased sex ratio.	Weston *et al.* (2004)*
Thinornis rubricollis	P2/P8 + Hae III	Blood	Agarose Gel (conc^{n} unknown)	0	Behaviour	Display behaviour	Display is carried out by both sexes, most often males.	Weston *et al.* (2005)*
Tringa glareola	P2/P8	Feather	2% Agarose Gel	5.6 (96% ethanol); 53.6 (dry feather)	Three repetitions	Migration	Temporary resident males migrate earlier than females.	Muraoka *et al.* (2009)
Tringa glareola	P2/P8	Blood	3.5% Agarose Gel	Not stated	None	Migration	Males arrive at stopover and breeding grounds earlier than females.	Remisiewicz *et al.* (2006)
Vanellus vanellus	2550F/ 2718R	Blood	2.2% Agarose Gel	0	Gonad analysis	Breeding	No correlation between egg size and sex of chick.	Lislevand *et al.* (2005)
Vanellus chinensis	2550F/ 2718R	Blood	Agarose Gel (conc^{n} unknown)	0	Behaviour	Breeding	Reproductive behaviour and social structure in this species.	Saracura *et al.* (2008)*
Vanellus cinereus	2550F/ 2718R	Blood	3% Agarose Gel	0	Mated pairs sampled	Sexual dimorphism	Males have significantly longer carpal spur length than females.	Wakisaka *et al.* (2006)*

3 Does sex-biased offspring survival predict sex-biased parental care roles?

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Abstract

Adult sex ratios (ASRs) can influence courtships, mating systems and parental care, according to theoretical models and empirical studies. However, *how* and *when* sex ratio variation emerges in wild populations is not clear. Biased offspring sex ratios may produce a biased ASR, and hence biased mating opportunities for males and females within a population. Here, we investigate sex ratios at hatching and subsequent offspring survival in four populations of Kentish plover *Charadrius alexandrinus* and snowy plover *C. nivosus*, two closely related shorebird species that exhibit different breeding systems across populations. We determined the sex of newly hatched chicks using genetic markers, and monitored their survival in two populations with largely male care and two populations with similar male and female parental roles. Extensive datasets were collected from each population over multiple years (2-5 years). Sex ratio at hatching did not differ from unity in any population. After hatching, in one population with largely male care, sons survived better than daughters in broods with uniparental care but not in broods with biparental care, whereas in one population where males and females care for offspring equally, females had better survival than males. We suggest that while the tendency for sex-biased offspring survival may lead to the production of biased adult sex ratios under certain environmental conditions, biased sex ratios may also develop after fledging in plovers. We call for further empirical studies to elucidate the link between sex ratios and the evolution of breeding systems across multiple populations with differing parental care and mating systems, but also caution that 'snapshot' studies disregarding temporal variation are likely to lead to errors in interpretation.

Author contributions

NdR: molecular sex-typing, statistical analysis, manuscript preparation
TS: field work, sample acquisition, statistical advice, manuscript improvement
CK: field work, sample acquisition, statistical advice, manuscript improvement
PLML and TB: manuscript improvement
AK: field work, sample acquisition, statistical analysis, manuscript improvement

Introduction

The evolutionary interests of males and females over reproduction often differ (Trivers 1972, Emlen and Oring 1977, Arnqvist and Rowe 2005). Determining the origins of sexual conflict is central to understanding the evolution of breeding systems (mating and parental care systems, Reynolds 1996). Parental care is costly to parents in both the short and long term, due to the metabolic costs and predation risk involved in caring for the offspring, as well as lost opportunities for mating (Clutton-Brock 1991; Balshine *et al.* 2002). These costs are paid by those that provide care, whilst the benefits of raising offspring successfully to independence are shared by both biological parents (Houston *et al.* 2005). It is in the interest of each parent to provide only as much care as is necessary for the survival of their offspring to independence, and for their partner to provide the majority of care. This is a major source of parental conflict. Among species with precocial young, one parent will often desert the family, leaving their partner to provide care alone (Lack 1968; Clutton-Brock 1991, Székely et al 1996; Kokko & Jennions 2008). Theoretical models suggest that the resolution of parental conflict is dependent on the mating opportunities available to males and females in a population. The sex with the greatest likelihood of finding a new mate is more likely to desert (Székely *et al.* 1999; McNamara *et al.* 2000; Jennions and Kokko 2010).

Mating opportunities are strongly influenced by adult sex ratios (ASRs), that is, the ratio of males to females in the adult population (Liker *et al.* 2013; Jennions and Kokko 2010). Biased ASRs can therefore produce biases in parental care roles and breeding systems (Colwell and Oring 1988, Kokko and Johnstone 2002, Ramsey 2010). Large variation in ASR is present in wild populations (Donald 2007, Székely *et al.* in prep.) though the causes of this variation are often unknown. ASR bias may arise in three major ways: firstly, as a result of biased sex allocation (West 2009), such that more embryos of one sex are produced than the other and this bias persists until adulthood; secondly, due to differential survival of males and females during either the embryonic, juvenile or adult stages (Liker & Székely 2005, Donald 2007); and thirdly, due to sex-biased dispersal, leading to a preponderance of one sex in certain locations (Eikenaar *et al.* 2010). Sex ratios are commonly established prior to adulthood (Donald 2007) and so investigating offspring sex ratios and juvenile survival can provide insights as to not only whether sex ratio bias exists, but how these biases arise within a population.

Previous studies into the influence of sex ratios on mating opportunities and breeding systems in single species have reported results consistent with theoretical expectations e.g. in Wilson's phalarope (*Phalaropus tricolor*; Colwell & Oring 1988),

dunnock (*Prunella modularis*, Davies 1992) and blue-headed vireo (*Vireo solitarius*; Morton *et al.* 2010). However, testing theoretical predictions in single species presents difficulties as most species do not exhibit sufficient variation in breeding systems and sex ratios.

In order to investigate the link between sex ratios and breeding system evolution in wild populations, we here investigate offspring survival across four populations of closely-related species with differing parental care strategies and mating systems. The Kentish and snowy plover (*Charadrius alexandrinus* and *C. nivosus*), until recently considered to be members of the same species (Küpper *et al.* 2009), are ground-nesting shorebirds with precocial offspring and flexible breeding systems (Lessells 1984; Warriner *et al.* 1986; Kosztolányi *et al.* 2009). Since plover chicks feed themselves after hatching, parental duties can be carried out by a single parent and so one parent often deserts their brood to mate again, producing up to four broods in a single breeding season (Lessells 1984, Warriner *et al.* 1986, Kosztolányi *et al.* 2011). All populations we investigated for the current study exhibit mixed parental care strategies including biparental, male-only and female-only care; however, in Ceuta (Mexico, snowy plovers) and Tuzla (Turkey, Kentish plovers), uniparental (single parent) brood care is largely carried out by males (hereafter termed 'male-biased care'), whereas in the other two populations, Al Wathba and Maio (Kentish plovers; United Arab Emirates and Cape Verde respectively), uniparental care is provided nearly equally by males and females (hereafter 'non sex-biased care'; Székely *et al.* 1999; Kosztolányi *et al.* 2009; Argüelles-Tico 2011; C. Küpper *et al.* unpublished data).

Kentish plovers in Turkey exhibit an extreme male-biased ASR (6.1: 1; Kosztolányi *et al.* 2011), and a substantial part of this skewed ASR is due to higher mortality among daughters than sons between hatching and fledging (Székely *et al.* 2004). This result is consistent with experimental assessment of mating opportunities that showed significantly shorter remating times for females than for males (Székely *et al.* 1999). Based upon theoretical models (Kokko & Jennions 2008) as well as the male-biased ASR and male-biased parental care identified in one plover population, we predict: a) that the sex ratio of chicks at hatching will not differ from parity in any population; b) that in populations exhibiting male-biased parental care survival of male chicks will be significantly better than for females between hatching and fledging (age 0-25 days), and that this will hold true among male and female siblings in the same family; c) that in populations with non sex-biased parental care there will be no sex differences in offspring survival; and d) that there will be no difference in the survival of chicks in

broods under the care of one or two parents (uniparental or biparental care) in any population.

Materials and methods

Field work

We monitored one population of snowy plovers at Bahía de Ceuta, Mexico (23° 54' N, 106° 57' W) from May to July 2006-2009, and three populations of Kentish plovers at: Tuzla Lake, southern Turkey (36° 42' N, 35° 03' E) from April to July, 1996 to 1999 and 2004; Al Wathba Wetland Reserve, United Arab Emirates (24° 15.5' N, 54° 36.2' E) between March and July, 2005 and 2006; and Maio Island, Cape Verde (15° 0.9' N, 23° 12' W) from September to November, 2008-2010.

In each population, a standard protocol was followed (see Székely *et al.* 2008) to search for nests by observation from a mobile hide, car or on foot. We predicted hatching dates based on the floating stage of eggs in lukewarm water, and visited nests daily as the expected hatching dates approached (Noszály & Székely, 1993). At first encounter after hatching, chicks were marked with a single metal ring and in most cases (>95%) an additional colour ring, and the length of the right tarsus was measured (to the nearest 0.1 mm). A droplet of blood (25 µl) was taken by puncturing the leg vein using a hypodermic needle for molecular sex-typing. Blood was stored in Eppendorf tubes containing 1 ml of Queen's Lysis Buffer (Seutin *et al.*1991).

Where chicks were captured away from the nest scrape and hatch dates were unknown, we estimated age based on tarsus length, since tarsi grow linearly between hatching and fledging (Székely & Cuthill, 1999). Growth parameters were estimated using ordinary least squares regression in R (package lmodel2) based on the tarsus length of chicks that were measured both on the day of hatching, and up to six times more before fledging (dos Remedios *et al.* submitted). We previously identified sex differences in growth at Ceuta and Tuzla (dos Remedios *et al.* submitted), therefore ages were estimated separately for males and females in these populations. Equations for age estimation were as follows:

Ceuta males: age (in days) = 2.659 x tarsus (mm) – 46.18

Ceuta females: age (in days) = 2.807 x tarsus (mm) – 48.26

Tuzla males: age (in days) = 2.190 x tarsus (mm) – 41.48

Tuzla females: age (in days) = 2.271 x tarsus (mm) – 41.95

Al Wathba: age (in days) = 1.730 x tarsus (mm) – 30.25

Maio: age (in days) = 1.812 x tarsus (mm) – 33.94

Parents were caught on the nest using funnel traps (Székely *et al.*, 2008) and colour ringed for identification from a distance. We observed broods with their parents up to the age of 25 days, since most chicks fledge at (or shortly after) this point (Székely & Cuthill 1999, Warriner *et al.* 1986). We monitored the number of chicks and parents present in each brood every two to three days, recapturing weekly to confirm the identity of survivors. Missing chicks that failed to reappear with the rest of their brood for the remainder of the field season were recorded as dead. Where entire families (including the parents that were last seen caring for the chicks) were lost, their fate was recorded as unknown. Chicks that were observed alive at the age of 25 days or older were considered to have successfully fledged.

In Ceuta and Tuzla, approximately 50% of captured chicks were experimentally cross-fostered, or their broods were reduced or expanded in number prior to hatching, for purposes outside the scope of this study. For all chicks, we recorded the identity of their 'genetic brood', the brood in which they were laid as eggs, and 'social brood', the brood in which they were raised, for inclusion in statistical analyses (see below).

Molecular sexing

DNA was extracted from blood samples using an ammonium acetate extraction method (Nicholls *et al.* 2000, Richardson *et al.* 2001). For molecular sex-typing, Z chromosome- and W chromosome-linked genes were amplified via the Polymerase Chain Reaction (PCR) using the Z-002B/Z-002D primers (Dawson 2007). For further verification, the W-specific Calex-31 primers, developed for Kentish plovers by Küpper *et al.* (2007) were utilised. PCR amplification was conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler under the following conditions: starting with 95^{o}C for 15 min, followed by 35 cycles of 94^{o}C for 30 s, 56^{o}C for 90 s, 72^{o}C for 60 s, and finishing with a final extension of 60^{o}C for 30 min. Amplicons were visualised on an ABI 3730 automated DNA analyzer. Fluorescent dye labelled primers separated the products of Z-002B/Z-002D primers into either one (ZZ) or two bands (ZW), indicating male or female, respectively. The W-specific *Calex-31* marker amplified in female samples only and was used to confirm sexing. Images were scored using GeneMapper software version 4.1 (Applied Biosystems). For 5% of samples in each population, PCR amplification and scoring were repeated a second time in a blind test.

Statistical analysis

Statistical analyses were performed in R (R Development Core Team, 2012). Data from each population were partitioned into three sets. Dataset A included only chicks from broods that were identified prior to hatching, in which all eggs hatched and all hatchlings were captured and sexed, to control for sex-specific embryo death. Dataset B consisted of dataset A and also chicks from broods where all hatchlings were caught and sexed and chicks from broods that were not identified prior to hatching, but which consisted of three chicks (the maximum clutch size observed in all studied populations) at the time of capture. Dataset C included all chicks that were captured and sexed in each population, regardless of time of capture or brood size (ie. including those in datasets A and B). Where any pair of parents produced multiple broods within or between breeding seasons, we included chicks from only one brood to avoid pseudoreplication. The included brood was that with the most chicks at hatching or, if broods contained equal numbers of hatchlings, the first brood for which data was collected.

Hatching sex ratio

The overall sex ratio in each population at hatching was assessed using the Wilcoxon signed rank test with dataset B, such that no broods were included in which any un-captured siblings may have died post-hatching. Additionally, the proportion of male chicks per brood was tested against the binomial distribution ($q = 0.5$) using chi-squared tests, taking into account the proportion of two chick and three chick broods in each population.

To test for seasonal differences in the sex ratio of hatchlings, separate Generalized Linear Mixed Models (GLMMs) for each population (dataset B) were fitted in R version 2.15.1 (R Core Team, 2012) with binomial error and logit function using package 'lme4' (version 0.999999-0). In each model, the response variable was sex, genetic brood identity was a random factor and either year (fixed factor), hatch date (fixed variable) or the pairwise interaction between year and hatch date were included in the models. We provide likelihood ratio test Chi-squared (χ^2) statistics with their probabilities (p).

Offspring survival

To investigate survival differences between individuals, Cox's proportional hazard mixed effect models (R package coxme) were fitted for each dataset per population separately. Explanatory variables were sex, hatch date, year, tarsus length, brood type

(single or mixed sex siblings) and the interactions of sex and each other variable. Social brood identity was a random factor. In Ceuta and Tuzla, experimental manipulations (among approximately 50% of chicks) were categorised based on expected survival effects and manipulation type (positive, negative, neutral or un-manipulated) was added as a factor in the coxme models, as well as the interaction term sex: manipulation. Manipulation type had no significant effect on survival in any case. The 'terminal' event in the models was death (see field work methods for classification). Individuals that reached the age of 25 days were classed as 'censored', as well as those in broods that were entirely lost prior to reaching 25 days old. The age of each chick last observation or capture was included as the final age in models. For each dataset per population, non-significant terms were removed from the full models, leaving only sex and the remaining significant terms in 'minimal' models. The significance of terms in the minimal models was assessed by likelihood ratio tests. In cases where all individuals from one or more years of study were censored, all data from those years was removed from the analysis (Tuzla A and B 1996, 1997 and 2004; Tuzla C 1996 and 2004; Maio A all years; Maio B 2007 and 2009; Maio C 2009). If year was not significant and was removed from the model, the remaining terms were tested based on the full dataset.

To test for differences in the survival of male and female siblings within the same brood, Wilcoxon signed rank tests were implemented. These included individuals in mixed sex broods of two or three chicks and data on their final age (age at last observation or capture).

Parental care

Coxme models were fitted to test the effects of care type (uniparental or biparental, i.e. single parent or two parent care) on survival within each population. This was only tested in Ceuta, Tuzla and Al Wathba since, in Maio, very few uniparental broods were observed and all chicks in these broods were of unknown/ censored fate. Coxme models included the number of days each chick survived after the date of desertion (if uniparental) or date of first capture (if biparental). Uniparental broods were included only if desertion occurred within the first half of the rearing period (≤ 12 days old). The maximum count for number of days survived in the models was 13 days, since this was the time it took for broods deserted by parents at 12 days old to reach fledging age (25 days old). Individuals that reached this point were classed as 'censored', as were individuals that went missing before this point (with fate unknown). The 'terminal' event in these models was death (see field work methods for classification). Only biparental broods in which no desertion occurred for the duration of the period

measured (13 days after first capture) were included. The explanatory variables were care type (uniparental or biparental), age (at desertion or first capture if biparental) and the interaction terms care type: sex, care type: hatch date, care type: year and care type: age (at desertion or first capture if biparental). Social brood identity was a random factor. Age and the interaction care type: age were included to test for potential age biases in the data, since younger chicks have higher mortality than older chicks, and biparental broods may have been 'first captured' at younger ages than the uniparental broods were 'deserted'. Likelihood ratio tests were performed to test the significance of terms in the coxme models.

Lastly, we tested the relationship between care type, chick sex and survival across populations using coxme models that additionally included species as a random effect. The results are included in Appendix 1.

Results

Sex ratio at hatching

Sex ratio at hatching did not differ significantly from parity in any of the four plover populations though in Al Wathba and Maio there was a marginally significant ($p < 0.1$) male-bias whereas in Tuzla there was a marginally significant female bias (Table 1; proportion male: Ceuta 0.519 ± 0.026, Tuzla 0.460 ± 0.023, Al Wathba 0.584 ± 0.049, Maio 0.596 ± 0.048). The distribution of males per brood at hatching did not differ from binomial in any population (Chi-squared tests; Table 1). However, in two populations hatchling sex ratio varied with hatch date: in Tuzla, the sex ratio early in the season was approximately 1:1 and became progressively female biased later in the season; in Al Wathba, the sex ratio early on was male-biased, reaching parity later in the season (GLMM; Table 2; Fig. 1).

Sex difference in chick survival

Sex differences in survival occurred in two out of four populations. In Tuzla a significant interaction appeared between sex and year - males survived better than females in 1999, females did better than males in 1998 and no sex differences were apparent in other years of study (based on datasets B and C; Table 3; Fig. 2). In Al Wathba, sex interacted significantly with hatch date – chick survival was higher earlier in the season than later on, and female chicks survived slightly better than male chicks in both cases (dataset A; Table 3; Fig. 2). No sex differences were present in Ceuta or Maio. Seasonal effects on offspring survival were present in all populations, with variation across the

season (hatch date; Tuzla, Al Wathba and Maio) and/ or between years (Ceuta, Tuzla and Maio; Table 3).

There was no difference in survival between male and female siblings within broods (Wilcoxon signed rank tests, Ceuta genetic broods: V = 688, p = 0.372; Ceuta social broods: V = 800, p = 0.836; Tuzla genetic broods: V = 1160.5, p = 0.896; Tuzla social broods: V = 1046, p = 0.971; Al Wathba: V = 79.5, p = 0.278; Maio: V = 46.5, p = 0.458).

Parental care analyses

Overall, there was no difference in the survival of chicks in broods with care from one parent (uniparental) or two parents (biparental). However in one population, Tuzla, survival was influenced by an interaction between care type and sex suggesting that male chicks survived better than female chicks in uniparental broods but not in biparental broods (Table 4; Fig. 3). Seasonal effects on survival also interacted significantly with care type in Tuzla, with early hatching chicks in uniparental brood surviving better than those in biparental broods or those hatching later in the season.

Discussion

Overall, sex ratios at hatching did not differ from unity in any of the four plover populations. We therefore suggest that, as predicted, sex ratios were not biased through sex allocation. After hatching, in the two populations where males and females take equal share in parental care (non sex-biased care), offspring survival was either not sex-biased (Maio), or was female-biased in relation to time of season (Al Wathba), in line with our predictions of equal sex ratios and equal mating opportunities for males and females. However, in Ceuta and Tuzla, where parental care is carried out mostly by males, our predictions were only partially supported. Male chicks survived better than daughters in Tuzla only within broods cared for by a single parent. No survival differences were found in Ceuta. We suggest that the tendency towards sex-biased survival may be present among plovers - in favour of sons in populations with male-biased care but females in populations with non-sex biased care – and that this may have a role in the development of sex ratio bias. We also detected an influence of environmental variables on sex-biased offspring survival, suggesting that environmental conditions might help to shape seasonal variation in sex bias. This bias

Table 1: Hatching sex ratio of plover chicks. Deviations from parity were tested in two ways: a) population sex ratios were tested using the Wilcoxon signed rank test and b) deviation from the expected binomial distribution of male chicks per brood was tested using Chi-square tests.

Population	Number of chicks (broods)	Hatchling sex ratio (proportion male)	Population sex bias (Wilcoxon test)[a]		Per brood sex bias (Chi-sq test of binomial distrib.)[b]	
		mean ± SE	V	p	χ^2	p
Ceuta, Mexico*	368 (159)	0.519 ± 0.026	35240	0.466	3.058	0.383
Tuzla, Turkey*†	489 (167)	0.460 ± 0.023	55125	0.078	3.078	0.380
Al Wathba, UAE	101 (47)	0.584 ± 0.049	3009	0.091	0.201	0.977
Maio, Cape Verde	104 (50)	0.596 ± 0.048	3255	0.050	4.516	0.211

*Male-biased care; †Extreme male-biased ASR

Table 2: Variation in hatchling sex ratio within and between years was tested using GLMMs with sex as the response variable and brood ID as a random factor. Values in bold indicate significant effects ($p < 0.05$).

Population	Number of chicks (broods)	Hatch date (GLMM)[c]		Year (GLMM)[c]		Hatch date x year (GLMM)[c]	
		χ^2	P	χ^2	p	χ^2	P
Ceuta, Mexico*	368 (159)	0.012	0.913	3.83	0.280	2.266	0.519
Tuzla, Turkey*†	489 (167)	**6.33**	**0.012**	0.682	0.409	0.232	0.630
Al Wathba, UAE	101 (47)	**4.47**	**0.034**	0.797	0.372	0.777	0.378
Maio, Cape Verde	104 (50)	0.536	0.464	0.305	0.581	0.112	0.738

*Male-biased care; †Extreme male-biased ASR

Table 3: Cox mixed effect models of survival among plover chicks, up to a maximum age of 25 days, based on age at last sighting. The significance of terms in 'minimal' models was assessed by likelihood ratio tests. Values in bold indicate significant effects ($p < 0.05$). *Male-biased care; †Extreme male-biased ASR.

Population	Data set	Number of chicks (broods)	Sex		Hatch date		Year		Sex x hatch date		Sex x year	
			χ^2 (df)	P	χ^2 (df)	p	χ^2 (df)	p	χ^2 (df)	P	χ^2 (df)	p
Ceuta, Mexico*	A	244 (84)	1.74 (1)	0.188	-	-	-	-	-	-	-	-
	B	368 (159)	0.89 (1)	0.346	-	-	-	-	-	-	-	-
	C	473 (225)	0.22 (1)	0.639	-	-	**9.71 (3)**	**0.021**	-	-	-	-
Tuzla, Turkey*†	A	384 (129)	0.13 (1)	0.721	-	-	**6.35 (1)**	**0.012**	-	-	-	-
	B	489 (167)	-	-	-	-	-	-	-	-	**4.81 (1)**	**0.028**
	C	645 (265)	-	-	-	-	-	-	-	-	**6.00 (1)**	**0.014**
Al Wathba, UAE	A	50 (18)	-	-	-	-	-	-	**4.21 (1)**	**0.040**	-	-
	B	101 (47)	0.05 (1)	0.818	**13.53 (1)**	**<0.001**	-	-	-	-	-	-
	C	176 (94)	0.52 (1)	0.471	**20.52 (1)**	**<0.001**	-	-	-	-	-	-
Maio, Cape Verde	A	46 (17)	0.86 (1)	0.354	-	-	-	-	-	-	-	-
	B	104 (50)	1.26 (1)	0.261	**6.54 (1)**	**0.011**	-	-	-	-	-	-
	C	299 (179)	0.50 (1)	0.478	**7.20 (1)**	**0.007**	**13.58 (2)**	**0.001**	-	-	-	-

Table 4: Survival of chicks in broods with uniparental or biparental care in three populations: Ceuta, Tuzla and Al Wathba Likelihood ratio tests were performed to test the significance of terms. Values in bold indicate significant effects ($p < 0.05$).

Population	Dataset	Number of chicks (broods)	Care type		Care type: hatch date		Care type: sex		Age		Care type: age	
			χ^2 (df)	p	χ^2 (df)	P	χ^2 (df)	p	χ^2 (df)	p	χ^2 (df)	p
Ceuta, Mexico*	A	244 (84)	0.05	0.825	0.398	0.528	0.05	0.826	0.62	0.430	0.04	0.837
	B	368 (159)	0.12	0.734	0.15	0.701	0.14	0.712	0.31	0.577	0.03	0.862
	C	473 (225)	0.01	0.931	1.31	0.253	0.04	0.833	0.03	0.868	0.41	0.523
Tuzla, Turkey*†	A	384 (129)	0.75	0.387	**3.91**	**0.048**	**4.27**	**0.039**	3.16	0.075	0.61	0.436
	B	489 (167)	0.73	0.393	**4.54**	**0.033**	**4.65**	**0.031**	2.80	0.094	0.43	0.514
	C	645 (265)	1.63	0.202	0.36	0.546	**5.58**	**0.018**	**3.89**	**0.048**	<0.01	0.972
Al Wathba, UAE	A	50 (18)	0.63	0.427	NA	NA	NA	NA	0.90	0.342	NA	NA
	B	101 (47)	0.79	0.374	2.44	0.118	2.29	0.130	0.12	0.732	0.56	0.453
	C	176 (94)	<0.01	0.978	3.75	0.053	0.694	0.405	0.06	0.799	0.36	0.548

*Male-biased care; †Extreme male-biased ASR.

Figure 1: Hatchling sex ratio variation across the season in a) Ceuta*, b) Tuzla*†, c) Al Wathba and d) Maio. Data were divided into seven intervals and the proportion of male hatchlings (mean ± SE) per interval are plotted ('jitter') as well as raw data ('rug', male = 1, female = 0). *Male-biased care; †Extreme male-biased ASR.

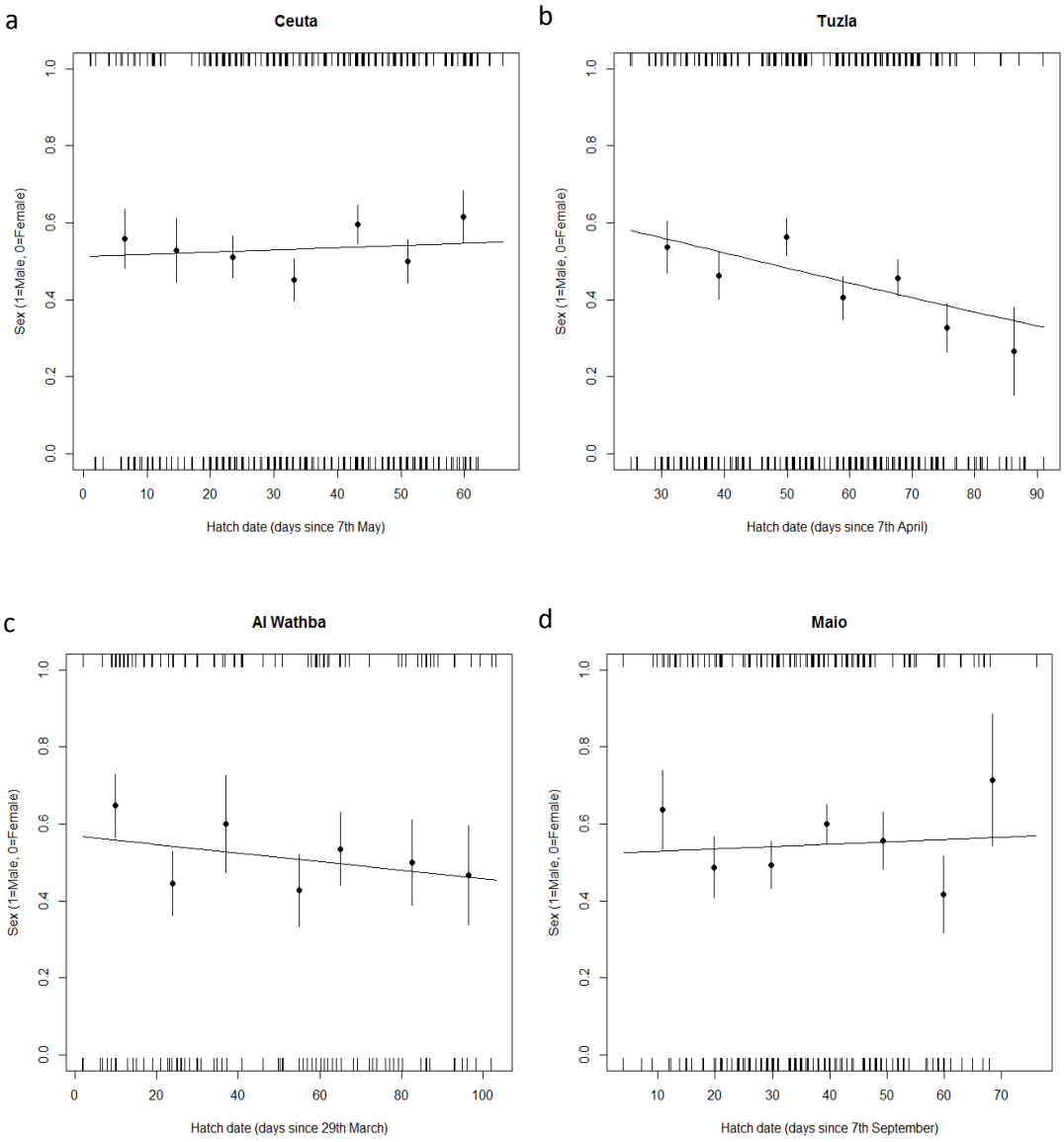


Figure 2: Interaction effects on survival of plover chicks for a) sex x year (Tuzla) and b) sex x hatch date (Al Wathba).

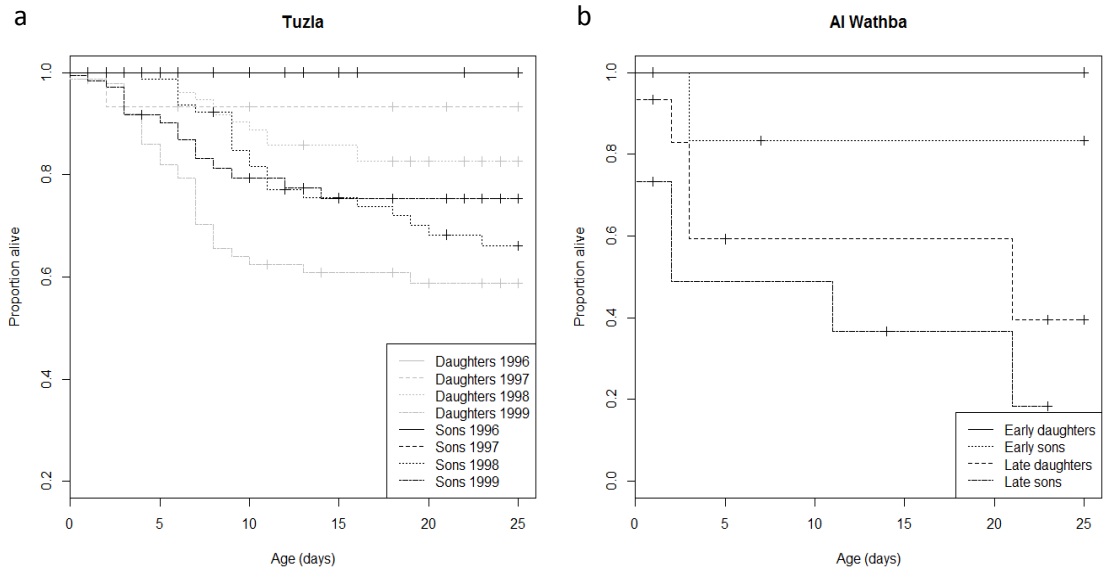
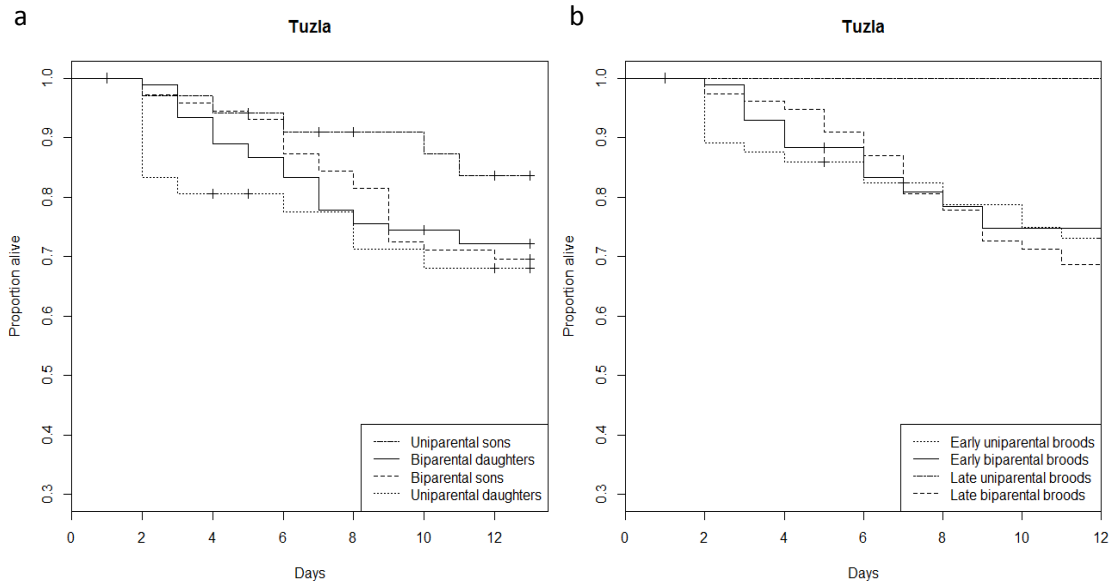


Figure 3: Kaplan-Meier survival curves for a) care type x sex and b) care type x hatch date in Tuzla.



may in turn bias mating opportunities, leading the overrepresented sex to become the main care giver in the population. However, since the observed sex-biases in offspring survival were not strong, this is not likely to be the only factor involved.

Since recent theoretical studies, as well as empirical studies on single populations, have strongly supported the link between sex ratio bias and biased parental care (McNamara *et al.* 2000; Jennions and Kokko 2010; Liker *et al.* 2013), we expected to observe male-biased survival across both populations with male-biased care. However, no sex bias was observed among offspring in Ceuta despite strongly male-biased parental care (Warriner *et al.* 1986; Argüelles-Tico 2011) and, in Tuzla, no strong overall sex differences in survival appeared. There may be four explanations for this: firstly, sex-biased survival may occur after fledging rather than among juveniles; secondly, sex-biases may arise in dispersal, such that females are less likely to return to their natal area to breed; thirdly, adult mortality may be higher among females than males as reported for snowy plovers in California, USA (Stenzel *et al.* 2011); or fourthly, parental care may not be fully explained by sex ratios in this species and male-biased care may have arisen due to males being better care-givers than females, or as a result of sexual selection. For example, among fish species such as the sand goby (*Pomatoschistus minutus*) and the fifteen-spined stickleback (*Spinachia spinachia*), females mate preferentially with more caring males, while providing no care themselves (Forsgren, 1997; Östlund & Anhesjö 1998). The fourth hypothesis, however, is unlikely to explain male-biased care on its own since in Tuzla, an extreme male-biased adult sex ratio has been identified (Kosztolányi *et al.* 2011) and in Ceuta, though ASR has not been directly measured, data on the recruitment of juveniles to the mating population in their first and second years suggests that there is a bias towards higher male recruitment (of the 2006-2009 hatchlings, 38 males and 22 females were recruited as breeders up until 2012; C. Küpper *et al.* unpublished data). Additionally, a modest male-biased adult sex ratio (ASR) was recently reported in another population of snowy plovers in California (Stenzel *et al.* 2011), therefore it is possible that ASR bias is also present within the Ceuta population.

Since we have little evidence that this bias is established during the juvenile stages, further research will be necessary in order to determine exactly how and when sex ratio bias occurs among plovers. Sex-biased survival has been reported among adults in a range of species including the common toad (*Bufo bufo*; Loman & Madsen 2010), the Milne-Edwards sifala (*Propithecus Edwardsi*; Tecot et al. 2013) and the hazel grouse (*Bomasa bonasia*; Montadert & Leonard 2006). Furthermore, estimating survival in the first year of life is an important factor in modelling population dynamics and

understanding the evolution of reproductive strategies across taxa (Anders *et al.* 1998; Adams *et al.* 2006; McKim-Louder *et al.* 2013), therefore we suggest the post-fledging period as a future area of focus.

In Tuzla, male chicks tended to have higher survival than female chicks in uniparental broods, and also higher survival than chicks of either sex in biparental broods. The reasons for this are not obvious and it seems counter-intuitive that one parent would provide a better level of care than two. It may be that differences in environmental conditions and/ or parental quality are responsible since broods that hatched early in the season and were left with one parent caring survived better than those that hatched later on and biparental broods (Székely *et al.* 2004). Székely & Cuthill (1999) reported higher mortality among Kentish plover broods in Tuzla later in the season than earlier on. However, in this study based on a larger dataset, we identified no overall survival differences in relation to hatch date and, among biparental broods, no survival differences across the season. We therefore find no evidence that conditions overall were better earlier in the season than later on. Instead, we suggest that early in the season, mothers may have been more likely to desert broods that were located in areas of greater resource abundance (Kosztolányi *et al.* 2006), or alternatively they may have deserted higher quality, 'caring' fathers more than 'less caring' fathers. The conditions in these broods may have been better than for broods of other areas or under the care of 'less caring' fathers and mothers in biparental broods. The male-biased nature of these survival effects in early uniparental broods may have occurred for several reasons: firstly, because these broods contained a greater proportion of males than later in the season when the proportion of female hatchlings in the population increased (Székely *et al.* 2004), secondly, because these sons were somehow 'fitter' and had an intrinsically higher chance of survival than those in other broods, or thirdly, because these sons were better able to capitalise on the benefits of being in these broods, for example by growing at higher rates than females and so reducing their period of vulnerability to predation (Chapter 4).

Conclusions

In this study of four plover populations with differing breeding systems, no sex ratio bias was present at hatching in any population and we found only partial evidence for a link between sex-biased offspring survival and sex-biased parental care. Where present, sex biases were in the predicted direction – male-biased in a population with male-biased care but female-biased in a population with non-sex biased care. Since male-biased adult sex ratios or male-biased recruitment have been previously identified in both Tuzla and

Ceuta, where males provide the majority of brood care, we see no reason to refute the hypothesis that sex ratios influence mating opportunities and parental care in plovers. We suggest either that sex biases in offspring survival are accentuated under particular environmental conditions, and that these conditions were not consistent during the years of this study, or that sex ratio bias develops largely after fledging in plovers, either due to sex-biased survival or sex-biased dispersal. Clearly, further research will be necessary to clarify these issues. In order to fully elucidate the link between breeding systems and sex ratios, we call for more empirical studies on how and when adult sex ratio bias arises across multiple populations with differing parental care and mating strategies. Furthermore, we caution against 'snapshot' studies that may result in errors in interpretation, and suggest that research frameworks must incorporate data from across breeding seasons as well as across years to account for temporal variation in environmental conditions.

Acknowledgements

We thank numerous assistants including Istvan Szentirmai, Adam Lendvai, Thijs van Overveld, Medardo Cruz-Lopez, Lydia Lozano-Angulo, Iwan Fletcher and Araceli Argüelles-Tico for help with field work. We also thank the wetland reserves at Al Wathba, Tuzla Lake and Salina Porto Ingles, Maio, for making this work possible. This study is part of the PhD dissertation of N. dos Remedios, and was funded by the NERC and National Geographic. Molecular sexing was supported by NERC-Biomolecular Analysis Facility at the University of Sheffield. CK was supported by a Marie Curie IEF postdoctoral fellowship. AK was supported by the Hungarian Scientific Research Fund (OTKA K81953) and the Hungarian Academy of Sciences ('Lendület' LP2012-37/2012). Permits for fieldwork were provided by the national agencies in each country. Lastly, thanks to Michael Hutchinson for editorial assistance.

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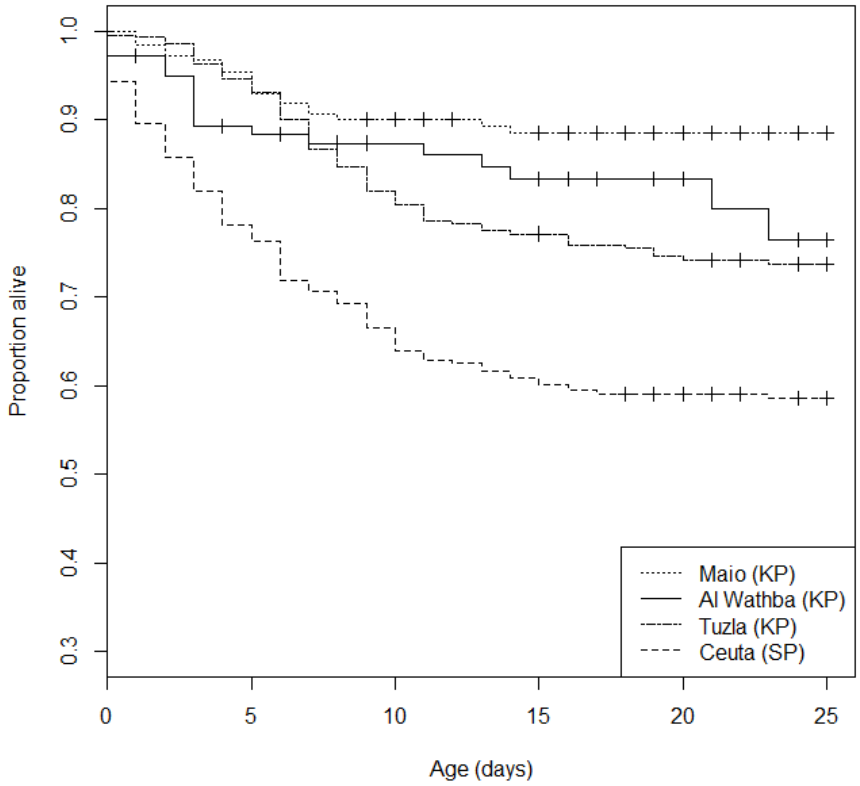
Appendix 1: Cross-species models

We implemented cross-species models to test for differences in offspring survival with parental care type per population and species, as well as sex. Cox's Proportional Hazard mixed effect models were implemented in R with explanatory variables: sex, care type, species and the interaction terms sex x care and sex x species. Response variables were age at last observation and chick fate. The 'terminal' event was death. Individuals that reached the age of 25 days were classed as 'censored', as well as those in broods that were entirely lost prior to reaching 25 days old. We provide likelihood ratio test Chi-squared (χ^2) statistics with their probabilities (p).

Table A1: Cross-population coxme models. Care type was classified as male-biased in Ceuta and Tuzla and non-sex biased in Al Wathba and Maio.

Predictor	**Dataset A**		**Dataset B**		**Dataset C**	
	χ^2 (df)	p	χ^2 (df)	p	χ^2 (df)	p
Sex	0.724	0.395	0.343	0.558	0.334	0.563
Care	1.272	0.259	0.075	0.785	1.597	0.206
Species	**5.184**	**0.023**	**12.415**	**<0.001**	**30.86**	**<0.001**
Sex x care	1.091	0.296	0.095	0.759	0.122	0.727
Sex x species	0.794	0.373	0.443	0.506	0.009	0.926

Figure A1: Kaplan Meier survival curves for the survival of plover chicks in each population (based on dataset C). KP = Kentish plover, SP = snowy plover. Snowy plover chicks have significantly greater mortality than Kentish plover chicks.



4 Does sex-biased offspring development predict sex-biased parental care roles?

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Abstract

Adult sex ratios (ASRs) take a prominent role in modulating social behaviour. Theory suggests that ASRs influence courtships, mating systems and parental care and that ASR bias often arises due to differential survival during juvenile stages. One rarely tested explanation for how ASR bias evolves is that offspring of one sex develop more slowly, remaining vulnerable to predation and detrimental conditions for longer than the opposite sex. Here we investigate offspring growth in relation to parental care roles in four populations of small plovers (Kentish plover *Charadrius alexandrinus* and snowy plover *C. nivosus*). Both species exhibit variable parental care strategies across populations. Based on previous results from a single population, we predicted that in two populations with largely male care, female chicks would be smaller at hatching and/or develop more slowly than male chicks and so potentially prolong their vulnerable period. Conversely, no bias should be observed in two populations with similar male and female parental roles. As predicted, in one population with paternal care, male chicks showed faster growth after hatching than females, whereas in the other population, male chicks had longer tarsi than females at hatching. In contrast, in the two populations with more balanced care roles, no sex differences in growth were detected, or daughters grew more quickly than sons. We therefore propose sex-specific development as an important component of sex-biased offspring mortality. To understand the implications for ASR bias and the evolution of breeding systems we call for more studies of sex-specific growth and maturation.

Author contributions

NdR: molecular sex-typing, statistical analysis, manuscript preparation
TS: field work, sample acquisition, statistical advice, manuscript improvement
CK: field work, sample acquisition, statistical advice, manuscript improvement
PLML and TB: manuscript improvement
AK: field work, sample acquisition, statistical analysis, manuscript improvement

Introduction

Breeding systems (mating and parental care systems, Reynolds 1996) are the product of an on-going battle between the sexes over their competing reproductive interests (Kokko and Jennions 2008, Alonzo 2010). Understanding the ecological and life-history mechanisms that influence the evolution of monogamy and polygamy, and biparental and uniparental care, are essential to understanding breeding system evolution. Theoretical models have outlined a tight connection between breeding systems and sex ratios (Colwell and Oring 1988, Kokko and Johnstone 2002, Ramsey 2010). Adult sex ratio (ASR) bias leads to bias in the relative mating opportunities available for males and females. Where the care needs of the offspring do not constrain both parents to provide care, the rarer sex can take advantage of the higher mating rate by reducing their parental care provision and re-entering the mating pool, leaving their partner 'holding the babies' (Breitwisch 1989, Yamamura & Tsuji 1993, McNamara et al. 2000, Kokko and Jennions 2008). Accordingly, male-biased ASRs have been linked to male-biased parental care roles (i.e. the majority of parental care is provided by males rather than females), with higher mating opportunities for females leading to the desertion of offspring by mothers (Thomas et al. 2007, Jennions and Kokko 2010, Stenzel et al. 2011).

Understanding how biased ASRs develop in wild populations with sex-biased parental care roles is important in understanding the factors that drive sex role evolution. Biased ASRs often arise as a result of differential survival of offspring across a wide range of species (Cooch et al. 1997, Oddie 2000, Székely et al. 2004, Donald 2007). Offspring size and growth can be significant predictors of survival to independence and therefore sex-specific development may be a key factor in sex-biased offspring survival (Clutton-Brock et al. 1985, Biro et al. 2006). Males and females can differ in terms of both energy acquisition and expenditure and therefore, sex-specific development is common (Case 1978, Gebhardt-Henrich and Richner 1998, Hegyi et al. 2011).

Among birds with large sexual size dimorphism (SSD), the smaller sex has often been reported to have a survival advantage during the juvenile stages because the increased metabolic requirements of the larger sex increase the likelihood of starvation at times of low food abundance (Lack 1968, Griffiths 1992, Lemons 2013, though see Råberg et al. 2005 and Hegyi et al. 2011). However, among bird species with only slight or moderate SSD, the reverse pattern has been identified. The larger sex has the survival advantage (Bortolotti 1986, Oddie 2000, Hipkiss et al. 2002, Råberg et al. 2005, Rowland et al. 2007) because faster growing offspring move out of the initial 'vulnerable' period when predation risk is highest and start regulating their own body temperature earlier,

and larger individuals survive extreme temperatures better than smaller, slower growing individuals (Rhymer 1988, Viñuela and Bustamante 1992, Bosque and Bosque 1995).

Even in species without SSD, growth and development may be sex-specific due to: i) differences in foraging behaviour and food intake (Slagsvold et al. 1992, Schekkerman and Visser 2001); ii) sex-chromosome linked, recessive mutations (Godfrey and Farnsworth 1952, Sellier 2000); iii) maternal effects acting via differential levels of maternal yolk hormones (Sockman and Schwabl 2000, Müller 2012, Helle et al. 2013); or iv) differences in sensitivity to abiotic and biotic conditions such as lower food availability (Martins 2004) or susceptibility to parasites (Potti 1999, Potti et al. 2002).

Shorebirds are increasingly being studied as models of breeding system evolution due to the wide variation exhibited in mating systems and parental care strategies (Thomas et al. 2007, Liker et al. 2013). The Kentish plover (*Charadrius alexandrinus*) and the closely related snowy plover (*Charadrius nivosus*) are particularly suited to investigations of ecological and social influences on breeding system variation. Although male-only care is present in most populations of both species, populations vary in frequencies of male and female care and polygamy (Lessells 1984, Warriner et al. 1986, Kosztolányi et al. 2009). Chicks are precocial, leaving the nest scrape within 1-2 h after hatching, are able to survive with care from just one parent (Warriner et al. 1986), and their survival may be influenced by genetic diversity (Küpper et al. 2010). We recently showed that a strongly polyandrous population of Kentish plovers in Southern Turkey had one of the most male-biased ASRs reported among birds (6.1 M: 1 F, Kosztolányi et al. 2011), and the extreme bias persisted over several years. In this population, male-biased sex ratios arose as a result of the differential survival of chicks prior to fledging (Székely et al. 2004). Similarly, snowy plovers in California, USA, exhibit very high levels of male-only parental care, maternal desertion and polyandry (Warriner et al. 1986, Stenzel et al. 2011), and a male-biased ASR was reported here resulting from higher adult survival for males than females (0.734 ± 0.028 and 0.693 ± 0.030 respectively; Stenzel et al. 2011).

Here, we investigate sex-specific development among plover chicks in three populations of Kentish plover and one population of snowy plover. Snowy plover and Kentish plover are phenotypically similar, and were considered to be the same species until recently (Küpper et al. 2009). Both species display small SSD and male adults are marginally larger than females (<1 mm difference in tarsus length, Küpper et al. 2009). Though incubation of the eggs is always carried out by both parents, all study

populations exhibit mixed parental care strategies after hatching and one parent may desert the brood. Within each population, care strategies include biparental, male-only and female-only care (Argüelles-Tico 2011); however, in Ceuta (Mexico, snowy plovers) and Tuzla (Turkey, Kentish plovers), brood care is largely carried out by males (hereafter termed 'male-biased care'; see Table 1; A. Kosztolányi, unpubl. data), whereas in the other two Kentish plover populations, Al Wathba and Maio (United Arab Emirates and Cape Verde, respectively), care is provided nearly equally by males and females (hereafter 'non sex-biased care'; see Table 1; A. Kosztolányi unpubl. data, Kosztolányi et al. 2009). To our knowledge, this is the first study of sex-specific development across populations of closely related species with differing breeding systems.

Given that most studies in birds with only minor SSD have shown survival advantages for the larger offspring (Hipkiss et al. 2002, Råberg et al. 2005, Rowland et al. 2007), we expected that among plover chicks the larger sex would have the survival advantage. We therefore investigated the following non-mutually exclusive predictions: a) males are larger than females at hatching in populations with male-biased care but not in populations with non sex-biased care, and b) males grow at a higher rate than females between hatching and fledging in populations with male-biased care but not in those with non sex-biased care.

Materials and methods

Field work

We collected data from juvenile snowy plovers at Bahía de Ceuta, Mexico from May to July 2006-2009. Kentish plover populations were investigated at Tuzla Lake, southern Turkey from April to July, 1996 to 1999 and 2004; at Al Wathba Wetland Reserve, United Arab Emirates between March and July, 2005 and 2006; and on Maio Island, Cape Verde from September to November, 2008-2010.

Standard protocol was followed (see Székely et al. 2008) to search for nests by observation from a mobile hide, car or on foot. We predicted hatch dates based on the floating stage of eggs in lukewarm water, and visited nests daily as the expected hatch dates approached (Noszály and Székely 1993). On the day of hatching, the majority of chicks were marked with a single metal ring and in most cases (>95%) an additional colour ring, weighed (to the nearest 0.1 g), and the length of the right tarsus was measured (to the nearest 0.1 mm). Additionally, a droplet of blood (25 µl) was taken for molecular sex-typing, by puncturing the leg vein using a hypodermic needle (dos Remedios et al. 2010). Blood was stored in Eppendorf tubes containing 1 ml of Queen's

Lysis Buffer (Seutin et al. 1991). Some chicks with known hatch date and age were not captured on the day of hatching, but were captured, measured and blood was collected subsequently. We monitored broods up to the age of 25 days, since most chicks fledge at (or shortly after) this point (Székely and Cuthill 1999). We attempted to measure growth of chicks multiple times prior to fledging. Chicks were recaptured during opportunistic encounters in the field and body mass and tarsus length were recorded (details in Table 1). Where any pair of parents produced multiple broods within or between breeding seasons, we included chicks from only one brood to avoid pseudoreplication. The included brood was that with the most chicks at hatching or, if broods contained equal numbers of hatchlings, the first brood for which data was collected.

Molecular sex-typing

DNA was extracted from blood samples using an ammonium acetate extraction method (Nicholls et al. 2000, Richardson et al. 2001). For molecular sex-typing, Z chromosome- and W chromosome-linked genes were amplified via Polymerase Chain Reaction (PCR) using the Z-002B/Z-002D primers and W-specific Calex-31 primers, (Dawson 2007, Küpper et al. 2007). PCR amplification was conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler under the following conditions: starting with 95^{o}C for 15 min, followed by 35 cycles of 94^{o}C for 30 s, 56^{o}C for 90 s, 72^{o}C for 60 s, and finishing with a final extension of 60^{o}C for 30 min. Amplicons with fluorescent dye labelled primers were visualised on an ABI 3730 automated DNA analyzer. PCR amplified products of the Z-002B/Z-002D primers were distinguished as either one (ZZ) or two products of differing size (ZW), indicating male or female, respectively. The W-specific Calex-31 primers amplified one product for female samples only. Markers were scored using GeneMapper software version 4.1 (Applied Biosystems).

Statistical analysis

i) Hatchling size

Separate Linear Mixed Models (LMMs) for each population were implemented in R version 2.15.1 (R Core Team, 2012) using package 'lme4' (version 0.999999-0) to analyse sex differences between chicks at hatching. The response variables were tarsus length or body mass of chicks captured on the day of hatching. Initial models included chick sex, year (fixed factors) and hatch date (fixed variable), with brood identity as a random factor. We also included pairwise interactions between sex, hatch date and year.

To account for differences in the timing of the breeding seasons, hatch dates were standardised using z-values for each year per population (z = (hatch date – mean hatch date) / standard deviation). We applied stepwise model selection based on likelihood ratio statistics, removing non-significant terms one by one, until the 'minimum model' for each population was reached, whilst still retaining chick sex in the model. We then used likelihood ratio tests to determine the significance of each term in the 'minimum model' (Chi-squared statistics are provided with their probabilities).

ii) Chick growth

Tarsus growth in plovers is well described by a linear equation (Székely and Cuthill 1999), and therefore standard major axis regression models were fitted for each population separately:

$T = a + b \times D$

where T is tarsus length (in mm) at age D (in days), and *a* and *b* are estimated parameters (size at hatching and growth rate respectively; see Table 1; see Supplementary Appendix 1 for plots of fitted lines). Standard major axis regression was used as both variables in the regression equation were random (i.e. not controlled) and subject to error.

Body mass growth was non-linear: after hatching there was an approximately five day lag period in body mass growth, during which chicks either lost mass or remained close to their hatching body mass, before beginning to gain mass. We compared five commonly-used growth models for best-fit across all populations using CurveExpert Professional 1.6.3 (Hyams 2012): Gompertz, logistic, von Bertalanffy, Ratkowsky and Morgan-Mercer-Flodin models (Gompertz 1825, Pearl and Reed 1923, von Bertalanffy 1938, Ratkowsky 1983, Morgan et al. 1975). Three-parameter models failed to capture the initial lag in body mass growth. The four-parameter Morgan-Mercer-Flodin (MMF) model had the best fit (i.e. lowest $SE_{Residual}$) and was therefore chosen as the most appropriate overall growth model for body mass:

$M = (a \times b + c \times D^d)/(b + D^d)$

where M is body mass (in g) at age D (in days), and *a*, *b*, *c* and *d* are estimated growth parameters (a: body mass at hatching; b: upper asymptote; c: growth rate; d: parameter that controls the point of inflection). Body mass growth parameters were estimated using the Levenberg-Marquardt algorithm (R package minpack.lm, see Table 1) for each population separately (see Supplementary Appendix 1 for plots of fitted curves).

The response variables for the LMMs, implemented in R for each population separately, were the residuals from the estimated growth models for tarsus length and

body mass. Residuals were consistent for individuals measured multiple times independent of age (i.e. chicks remained larger or smaller than average with age). In Ceuta, no recaptures were made in 2008, therefore no data from this year is included in the growth analyses. Initial models included chick sex, year (fixed factors) and standardised hatch date (fixed variable; see above), with both brood and chick identity as random factors. We also included pairwise interactions between sex, year and hatch date. In two populations (Ceuta and Tuzla) approximately 50% of chicks took part in experiments in which chicks were moved between broods before or shortly after hatching. Therefore we ran separate model sets with either social or genetic brood ID as random factors. The two model sets gave qualitatively the same results, therefore we report only the results of models with social brood ID. Model selection and testing of terms in the 'minimum model' were carried out as in the case of hatchling size (see above).

As the effect of sex on chick size and growth was tested multiple times in the four populations, we investigated whether the obtained significant p-values may be the by-product of multiple testing by calculating q-values (Storey 2002). The q-value analysis revealed that the estimated cumulated number of expected false positives among the significant sex effects (at $p \leq 0.05$) is 0.39, i.e. less than one case, therefore we concluded that the reported significant sex effects are likely to be truly significant. We also tested the relationship between care type, chick sex and hatchling size or growth across populations using LMMs that additionally included species as a random effect. The results are given as Supplementary material (Appendix 2, Table A1).

Results

i) Hatchling size

In Ceuta, male hatchlings had larger tarsi than female hatchlings ($\beta = 0.161 \pm 0.061$ (SE), Table 2) though there was no significant sex difference in body mass (Fig. 1). In Tuzla, male hatchlings were heavier at hatching than females in four years but not in one year (sex: year interaction, Table 2, Fig. 1.). Concurrently, in populations with non sex-biased care (Al Wathba and Maio), no sex differences in hatchling size were observed.

Hatch date and/or year predicted hatchling size in all four populations (Table 2). Body mass was larger among individuals hatching later in the season in all years in Tuzla and in three of four years in Ceuta (Tuzla $\beta = 0.113 \pm 0.038$; Ceuta year: hatch date interaction). Conversely, in Al Wathba tarsus length decreased over the season ($\beta = -0.631 \pm 0.120$) whilst in Maio body mass across the season varied significantly between

years, with considerably smaller hatchlings later in the season in one of three years (Fig. 1).

ii) Chick growth

Of the two populations with male-biased parental care roles, male chicks grew faster than females in Tuzla (tarsus $\beta = 0.473 \pm 0.178$; mass $\beta = 0.679 \pm 0.318$; Table3; Fig. 2), however in Ceuta, no sex differences were identified. Of the populations with unbiased care, no sex differences in growth were observed in Al Wathba although in Maio, tarsus growth was faster among females than males in late hatching broods during the latter half of the season (sex: hatch date interaction; Table 3; Fig. 2).

In Tuzla, chicks that hatched later in the season grew faster than those hatching earlier on (tarsus: $\beta = 0.257 \pm 0.107$; mass: $\beta = 0.589 \pm 0.184$; Table 3), and this was also the case for tarsus growth in Ceuta in two of three study years (2006 and 2009; year: hatch date interaction) though in one year (2007), body mass growth in Ceuta was faster among those hatching earlier in the season than later on. Hatch date predicted mass gain in Maio with early season hatchlings growing faster than late season hatchlings in two of the three years (Fig. 2; year: hatch date interaction).

Discussion

Our study provides some support for the predicted link between sex-biased offspring development, sex ratio biases and parental care strategies. Firstly, of the two populations where males provide the majority of brood care ('male-biased care'), sons either grew more quickly than daughters (Tuzla; tarsus and body mass) or were larger at hatching (Ceuta; tarsus length). In contrast, of the two populations where care is provided equally by mothers and fathers ('non sex-biased care'), sex differences were either not present (Al Wathba) or daughters grew more quickly than sons later in the season (Maio; tarsus only).

Table 1. Summary data and growth parameters for chicks in four plover populations (see Methods for growth equations). Tarsus growth parameters are, a: size at hatching and b: growth rate. Body mass growth parameters are, a: size at hatching; b: upper asymptote; c: growth rate; d: parameter that controls the point of inflection.

	Ceuta, Mexico	**Tuzla, Turkey**	**Al Wathba, United Arab Emirates**	**Maio, Cape Verde**
Location	23° 54' N, 106° 57' W	36° 42' N, 35° 03' E	24° 15.5' N, 54° 36.2' E	15° 0.9' N, 23° 12' W
Parental care at age 14 days (A. Kosztolányi, unpubl. data), proportion of broods (95% CI) where:				
Males care	0.988 (0.957 – 0.997)	0.933 (0.841 – 0.974)	0.805 (0.660 – 0.898)	0.825 (0.706 – 0.902)
Females care	0.175 (0.124 – 0.240)	0.617 (0.490 – 0.729)	0.732 (0.581 – 0.843)	0.842 (0.726 – 0.915)
n (broods)	166	60	41	57
Number of chicks measured on day of hatching, broods	262, 122	347, 144	70, 39	80, 51
Years of study	2006-2009	1996-1999, 2004	2005, 2006	2008-2010
Number of chicks measured between 1 and 25 days old	128	115	33	41
Mean number of captures per chick	2.1	2.3	2.0	1.6
Tarsus length (mm) at hatching (mean ± SE):				
Males	17.60 ± 0.05	19.05± 0.06	18.52 ± 0.16	19.61 ± 0.18
Females	17.35 ± 0.05	18.92 ± 0.05	18.09 ± 0.17	19.47 ± 0.14
Body mass (g) at hatching (mean ± SE):				
Males	6.16 ± 0.04	6.26 ± 0.04	6.62 ± 0.09	6.97 ± 0.16
Females	6.20 ± 0.03	6.18 ± 0.04	6.63 ± 0.07	6.62 ± 0.11
Tarsus growth parameters:				
a	17.422	18.847	17.643	18.957
b	0.348	0.440	0.558	0.498
Fit of linear growth model: r^2	0.818	0.871	0.875	0.815

(Continued...)

Body mass growth parameters:				
a	6.07	6.17	6.11	6.88
b	6034	992.77	1686	46600
c	25.42	33.30	35.18	19.87
d	3.02	2.41	2.64	4.45
Fit of MMF growth model: r^2	0.762	0.863	0.953	0.856

Table 2. Tarsus length and body mass of plover chicks at hatching. The significance of terms in 'minimum' Linear Mixed Models was assessed by likelihood ratio tests. Values marked with * indicate significant effects ($p \leq 0.05$). Main effects were only tested if they were not in significant interaction with other variables.

Response	Predictor	Ceuta, Mexico		Tuzla, Turkey		Al Wathba, UAE		Maio, Cape Verde	
		χ^2 (df)	P	χ^2 (df)	P	χ^2 (df)	p	χ^2 (df)	p
Tarsus length	Sex	6.94 (1)	0.008*	1.06 (1)	0.324	0.33 (1)	0.568	0.37 (1)	0.544
	Hatch date					21.07 (1)	<0.001*		
	Year	12.59 (3)	0.006*						
Body mass	Sex	0.38 (1)	0.540			0.33 (1)	0.569	<0.01 (1)	0.987
	Hatch date			8.65 (1)	0.003*				
	Sex × Year			10.78 (4)	0.029*				
	Year x Hatch date	11.01 (3)	0.012*					16.04 (2)	<0.001*

Table 3. Tarsus and body mass growth of plover chicks. Response variables in Linear Mixed Models (LMMs) were the residuals from the estimated growth models for tarsus or body mass (linear or Morgan-Mercer-Flodin models respectively). The significance of terms in 'minimum' LMMs was assessed by the likelihood ratio test. Values marked with * indicate significant effects ($p \leq 0.05$).

Response	Predictor	Ceuta, Mexico		Tuzla, Turkey		Al Wathba, UAE		Maio, Cape Verde	
		χ^2 (df)	P	χ^2 (df)	p	χ^2 (df)	P	X^2 (df)	p
Tarsus length	Sex	0.72 (1)	0.396	6.74 (1)	0.009*	1.74 (1)	0.187		
	Hatch date			5.19 (1)	0.023*				
	Sex x Hatch date							4.40 (1)	0.036*
	Year x Hatch date	7.68 (2)	0.022*						
Body mass	Sex	0.05 (1)	0.827	4.39 (1)	0.036*	0.95 (1)	0.331	0.09 (1)	0.760
	Hatch date			9.28 (1)	0.002*				
	Year								
	Year x Hatch date	6.68 (2)	0.035*					9.95 (2)	0.007*

Figure 1. Size of male and female hatchlings in four plover populations: a) tarsus length and b) body mass. Points represent predicted values (with standard errors) from the minimal models (see Table 2). Separate plots are provided to visualize the effects of significant environmental variables, namely 'Year', 'Hatch date' and their interactions. Note that hatch date was a continuous variable in the analyses, however, it has been converted to a binary variable to visualize interactions.
◆ male; ◇ female; ■ male (early season); □ female (early season); ▲ male (late season); △ female (late season)

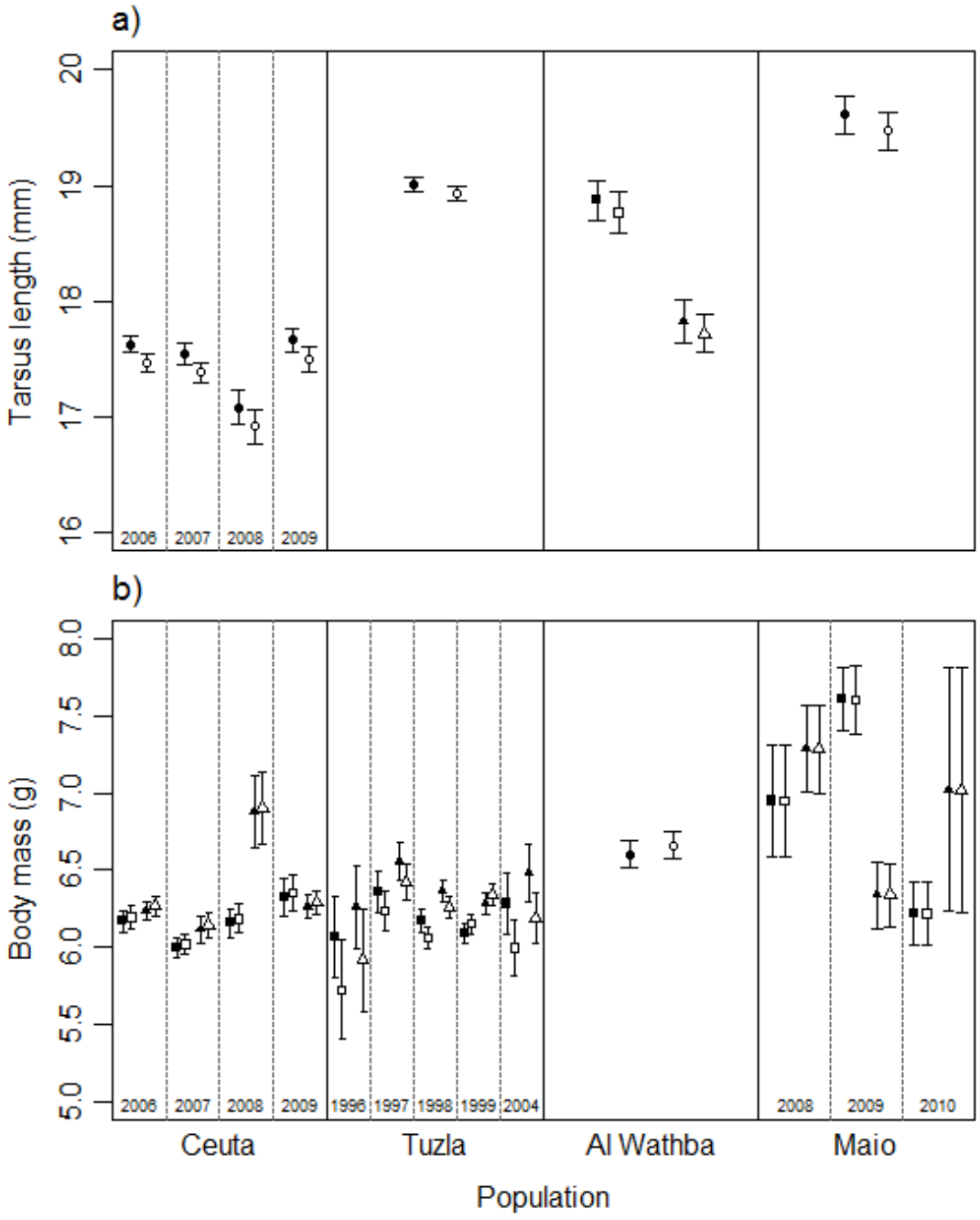

Figure 2. Growth of male and female chicks in four plover populations for a) tarsus and b) body mass. Residuals are from the growth models estimated

male; female; male (early season); female (early season); male (late season);

separately for each population. Points represent predicted values (with standard error) from the minimal models (see Table 3). Separate plots are provided to visualize the effects of significant environmental variables, namely 'Year', 'Hatch date', and their interactions. Note that hatch date was a continuous variable in the analyses, however, it has been converted to a binary variable to visualize interactions.

Implications of sex-specific growth

In populations where male hatchlings are larger in body size or grow faster than females, male offspring are likely to have a survival advantage over females, as previously reported (Råberg et al. 2005, Rowland et al. 2007) and this may precipitate into male-biased sex ratios, although growth is probably not the only factor involved. Male-biased adult sex ratios have been previously implicated in producing female-biased mating opportunities, encouraging desertion of broods by mothers with a strong chance of mating again quickly, such that parental care is left to the fathers (Székely et al. 1999, Donald 2007, Kokko et al. 2008, Kosztolányi et al. 2011).

Given that male-biased offspring development occurred in only two of the four populations studied, it must be questioned why this was not the case in all populations. Firstly, small samples sizes can sometimes result in an inability to reject the null hypothesis. In this study, sample sizes were lower for the two populations with non-sex biased parental care roles than for those with male-biased care roles, however, a sex effect was identified in one of the populations with smaller sample size (Maio, Table 2, Fig. 2). Additionally, the standard errors of the fitted values in the populations with smaller sample sizes were in several cases of comparable magnitude with those in populations with larger sample sizes (see Figs 1 and 2). It is therefore unlikely that small sample sizes were responsible for the observed results.

Secondly, our results cannot be explained by phylogenetic or population genetic differences as Kentish plovers display high levels of gene flow across geographic distances up to 10,000 km (Küpper et al. 2012). Kentish plovers breeding in Tuzla are genetically most similar to the Al Wathba population, and yet plovers in Tuzla exhibit greater similarity to snowy plovers in terms of both breeding system and male-biased growth. These results are in line with recent analyses of incubation behaviour across ten plover populations (Vincze et al. 2013), suggesting that environmental differences between populations are more important to sex-specific development and breeding systems than shared recent evolutionary history.

Thirdly, ambient environment may have a major impact on energy expenditure as chicks must continually adjust resource allocation based on their present needs (O'Connor 1977, Boag 1987, Janssen et al. 2011). For example, with extreme ambient temperatures, chicks need to invest more energy in thermoregulation (Visser 1998); lower rainfall decreases food availability so foraging time will need to increase (Schew and Ricklefs 1998); a high abundance of parasites requires more investment in immune response (Chandra and Newberne 1977, Møller and Saino 1998); and higher levels of

predation will require chicks to expend more energy fleeing to survive (Pravosudov and Lucas 2001). Therefore, there is a network of energy trade-offs such that, when survival pressures are high, resources will be preferentially allocated towards components of immediate functional priority, rather than growth (O'Connor 1977, Case 1978, Øyan and Anker-Nilssen 1996).

We suggest male and female plover chicks differ in their response to environmental selection pressures, and that the pressures may be higher in Ceuta and Tuzla, populations where males provide the majority of brood care, than in Al Wathba and Maio, where mothers and fathers provide equal levels of care. The seasonal effects we identified across all populations (both within and between years) support an environmental influence on hatchling size and offspring growth, which may in turn influence chick survival. However, since the populations were located on different continents, at different latitudes and with greatly differing habitats (i.e. island, coastal or inland salt marsh), further research will be necessary to identify the environmental pressures at these locations.

Causes of sex-specific growth

Sex-specific development in plovers may be caused by one or multiple factors. The disparity in the stage at which sex differences in body size occur in the two populations with male-biased uniparental care makes it difficult to identify these factors specifically here. In Ceuta, males are larger than females in tarsus length at hatching therefore sex differences may be the result of maternal effects (e.g. differential allocation of maternal yolk hormones or nutrients; Andersson et al. 2004, Sockman et al. 2008) or detrimental recessive mutations on the Z-chromosome (since females are the heterogametic sex, ZW; Haldane 1922, Turelli and Orr 1995, Mank et al. 2010) which may be expressed under particular, harsh environmental conditions. For example, we previously found that heterozygosity at one conserved autosomal microsatellite marker was a good predictor of survival chances of female Kentish plover chicks (Küpper et al. 2010). However, in Tuzla there were no significant overall differences in the size of males and females at hatching. Instead, growth rates of tarsus and body mass were higher for males than females after hatching, therefore alternative factors are implicated such as differences in foraging behaviour (e.g. foraging duration, location, technique and diet; Charnov 1982, Michler et al. 2010) or differences in energy allocation after hatching. For example, females often invest more in immunity than males as their priority is to increase longevity, whereas males invest more in increasing their mating success (Bateman 1948,

Trivers 1972, Rolff 2002) - if this process begins early in life and female growth is reduced as a result, female chicks may have a short-term survival disadvantage in terms of defence from predation (i.e. slower escape) in populations where predation pressure is high.

Conclusions

In summary, we present novel empirical evidence for male-biased offspring development in populations where males provide the majority of parental care. Male-biased offspring survival is implicated in the generation of male-biased adult sex ratios among plovers, producing female-biased mating opportunities leading to brood desertion by females. We propose that male-biased growth is an important component of male-biased offspring survival. Furthermore, since growth rates vary significantly both within and between seasons, as well as between populations, we suggest that sex differences are strongest when survival pressures are high. Future studies should determine the factors responsible for intrinsic sex differences in development, in order to further elucidate the mechanisms by which parental care strategies and mating systems evolve.

Acknowledgements

We thank numerous assistants including Istvan Szentirmai, Adam Lendvai, Thijs van Overveld, Medardo Cruz-Lopez, Lydia Lozano-Angulo, Iwan Fletcher and Araceli Argüelles-Tico for conducting field work. We also thank the wetland reserves at Al Wathba, Tuzla Lake and Salina Porto Ingles, Maio, for making this work possible. This study is part of the PhD dissertation of N. dos Remedios, and was funded by the NERC and National Geographic. Molecular sexing was conducted at NERC-Biomolecular Analysis Facility at the University of Sheffield. CK was supported by a Marie Curie IEF postdoctoral fellowship. AK was supported by the Hungarian Scientific Research Fund (OTKA K81953) and the Hungarian Academy of Sciences ('Lendület' LP2012-37/2012). Permits for fieldwork were provided by the national agencies in each country. Lastly, thanks to Michael Hutchinson for editorial assistance.

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Appendix 1: Growth models

Figure A1. Tarsus length of chicks monitored between hatching and fledging across four plover populations: a) Ceuta, b) Tuzla, c) Al Wathba and d) Maio. For each population, the estimated of linear growth line is plotted based on standard major axis regression models (r^2 values are provided in Table 1).

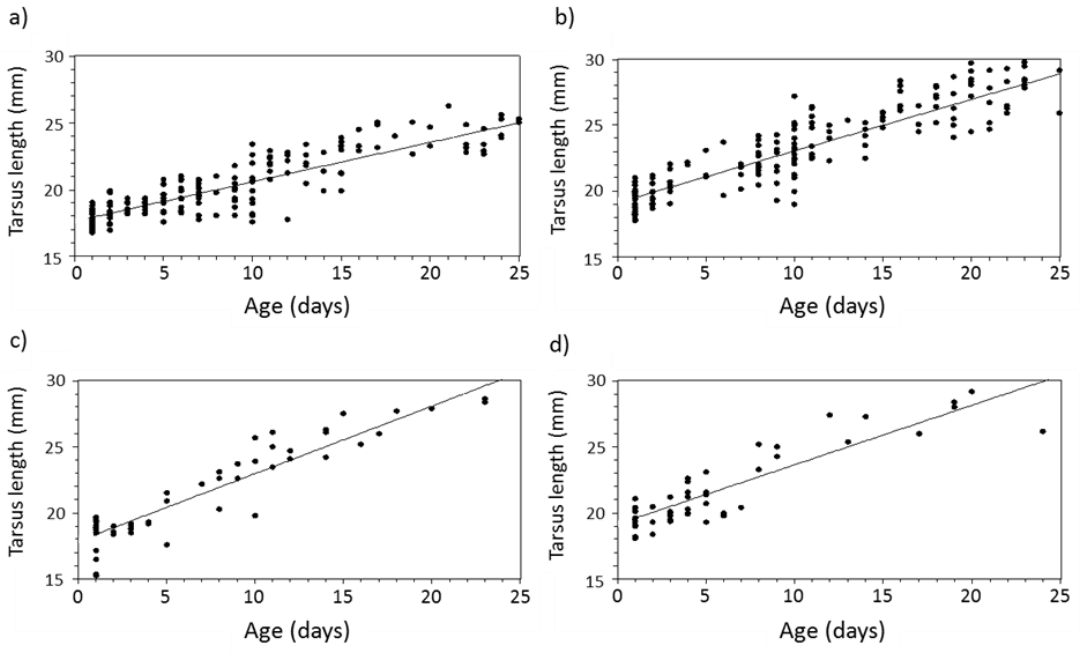
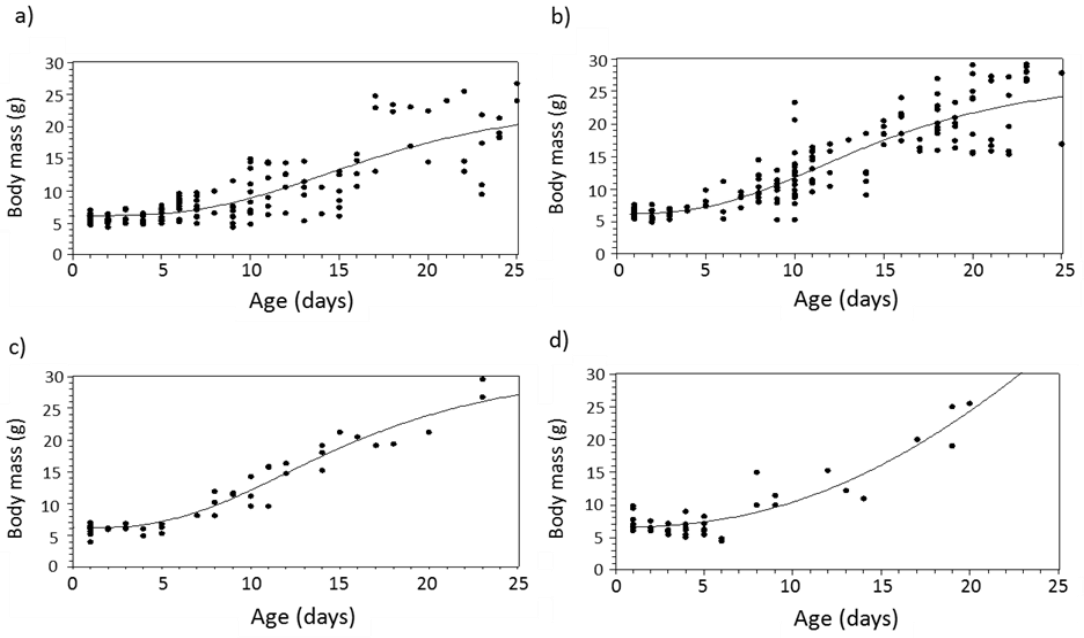


Figure A2. Body mass of chicks monitored between hatching and fledging across four plover populations: a) Ceuta, b) Tuzla, c) Al Wathba and d) Maio. For each population, the estimated body mass growth curve is plotted based on 4-parameter Morgan-Mercer-Flodin (MMF) models (r^2 values are provided in Table 1).



Appendix 2: Cross-species models

We implemented cross-species models to test for differences in chick growth with parental care type and species, as well as sex. Linear mixed effect models (LMMs) were implemented in R. For hatchling models, the response variable was tarsus length or body mass, chick sex and care type were fixed factors, and brood identity and species were random factors. For growth LMMs, growth parameters (linear and Morgan-Mercer-Flodin models for tarsus and mass growth, respectively) were estimated based on all chicks (tarsus parameters: a = 0.39, b = 18.31; mass parameters: a = 6.19, b = 3012, c = 28.10, d = 2.94) using Standard Major Axis regression (tarsus length; R package smatr) or Levenberg-Marquardt algorithms (body mass; R package minpack.lm), and the residuals from the estimated models were used as response variables. In each LMM, care type and sex were fixed factors, and brood identity, chick identity and species were random factors.

Table A1. Hatchling size and growth (response variables) across four plover populations. The significance of variables was assessed by likelihood ratio tests. Values in bold indicate significance levels of $p \leq 0.05$.

Response	Predictor	Hatchling size		Growth	
		χ^2 (df)	p	χ^2 (df)	P
Tarsus length	Sex	**5.125 (1)**	**0.024**	**7.593 (1)**	**0.006**
	Care type	0.046 (1)	0.826	**5.052 (1)**	**0.025**
	Species	**203.070 (1)**	**<0.001**	**92.597 (1)**	**<0.001**
Body mass	Sex	0.059 (1)	0.808	2.584 (1)	0.108
	Care type	**50.480 (1)**	**<0.001**	<0.001 (1)	>0.999
	Species	<0.001 (1)	>0.999	**18.013 (1)**	**<0.001**

5 Exploring the global origins of the genus *Charadrius*: a phylogeographic study based on nuclear and mt DNA

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Abstract

Where and when did widespread taxa evolve? Establishing phylogenetic relationships within a clade can help to infer ancestral origins and identify factors associated with species radiation. The small plovers, genus *Charadrius,* are cosmopolitan shorebirds, distributed across all continents except Antarctica. Here we present a global, species-level molecular phylogeny of this group based on four nuclear (*ADH5*, *FIB7*, *MYO2* and *RAG1*) and two mitochondrial (*COI* and *ND3*) genes, and use the phylogeny to examine the biogeographical origin of the genus. A Bayesian multispecies coalescent approach with incorporated mitochondrial molecular clock identified the genus as monophyletic, consisting of two major clades (*CRD I* and *CRD II*) that diverged 79.6–48.6 million years ago. Clade *CRD I* contains three species (*Thinornis novaeseelandiae, Thinornis rubricollis* and *Eudromias morinellus*), and *CRD II* one species (*Anarhynchus frontalis),* that were previously placed outside the *Charadrius* clade. In contrast to earlier work, ancestral area analyses using parsimony and Bayesian methods consistently supported an origin of the *Charadrius* plovers in the Northern hemisphere. Based on this and molecular clock data, we propose that major radiations in this group were associated with shifts in the range of ancestral species in response to global cooling during the Oligocene (*CRD I*) and Miocene (*CRD II*) periods, driving some taxa into the Southern hemisphere.

Author contributions

NdR: sample acquisition, DNA extraction, sequence analysis, phylogenetic and statistical analyses, manuscript preparation
CK: sample acquisition, phylogenetic advice, manuscript improvement
PLML – sample acquisition (museum specimens), DNA extraction (museum specimens), phylogenetic advice, manuscript improvement
TB: manuscript improvement
TS: sample acquisition, manuscript improvement

Introduction

Within a monophyletic clade all species are derived from a single ancestor that originated in a particular geographic area. Determining how widespread species attained their current distributions provides insights into the ultimate drivers of species radiation and range changes (Avise, 2009; Edwards *et al.,* 2012). In addition, studying the history of species' distributions can help to model species' responses to future climate changes. Climatic oscillations have played a major role in past species radiations, extinctions and changes of distribution (Blondel and Mourer-Chauviré, 1998; Hewitt, 2004). Determining how certain species survived these oscillations is important in determining future population viability in the face of climate change. Molecular phylogenies can provide the means for both timing species diversification events (Kumar, 2005; Drummond *et al.,* 2006; Weir and Schluter, 2008; Jetz *et al.,* 2012) and estimating their geographic origins (Schweizer *et al.,* 2011; Yu *et al.,* 2013) although establishing the exact phylogenetic relationships between species within a clade is often challenging.

The history of phylogenetic inference for the *Charadriiformes* (shorebirds, gulls and alcids) provides an example of such challenges. This clade provides ideal study organisms for many areas of research since the taxa exhibit remarkable diversity in breeding systems, migratory behaviours, modes of offspring development, sexual size dimorphism, egg size and plumage colouration (Graul, 1973; Piersma and Wiersma, 1996; Thomas *et al.,* 2007; Delany *et al.,* 2009). Comparative analyses have made heavy use of existing phylogenies (Székely *et al.,* 2004a; Lislevand and Thomas, 2006; Thomas *et al.,* 2006a, 2006b) despite the relationships between many species not being fully resolved (Thomas *et al.,* 2004; Baker *et al.,* 2012; Barth *et al.,* 2013; Corl and Ellegren, 2013).

Within the *Charadriiformes,* the genus *Charadrius* consists of 30 species of small plovers with highly diverse behavioural, ecological and life history traits, that breed on all continents except Antarctica. The phylogenetic history of the genus is controversial and, to date, molecular analyses have been based on only partially complete species datasets. The most complete molecular *Charadrius* phylogeny in terms of included taxa (26 species) was based on partial nuclear and mtDNA sequence data and outlined two major species clusters (Barth *et al.,* 2013). However, Barth *et al.* (2013) controversially suggested that the genus was paraphyletic due to the placement of genera *Vanellus, Phegornis, Anarhynchus, Thinornis* and *Elseyornis* within the *Charadrius* clade. This result was in stark contrast with traditional theories and phenotypic studies (Livezey, 2010),

but supported earlier work based on allozymes and *cyt b* variation in a small number of species (four *Charadrius* species, Christian *et al.* 1992; 10 *Charadrius* species, Joseph *et al.,* 1999). A limitation of the phylogeny presented by Barth *et al.* (2013) was the incomplete sampling of molecular markers (66% of sequences missing; 70% missing characters). This can be problematic since phylogenetic analyses that rely on patchy datasets with large areas of missing data can lead to erroneous tree topologies (Lemmon, 2009; Roure *et al.,* 2013) and therefore further analyses using more complete datasets are needed to more accurately establish the phylogenetic relationships within the genus.

The biogeographical origin of the *Charadrius* group is still debated. Contrasting Northern hemisphere and Southern hemisphere hypotheses have been proposed. Proponents of the Northern hemisphere hypothesis have focussed on phenotypic characters, suggesting that the primitive *Charadrius* stock had breast bands, black lore lines and crown patches similar to the modern Palearctic-breeding common ringed plover (*C. hiaticula*; Bock, 1958; Graul, 1973). They speculated that these ornaments were reduced as their descendants colonised habitats with lighter coloured substrates. Additionally, Northern hemisphere proponents suggested that the ancestral species produced clutches of four eggs (as do *C. hiaticula* and neighbouring Palearctic species) from which species with reduced clutch sizes of two or three eggs evolved (Maclean, 1972). In contrast, in support of the Southern hemisphere origin hypothesis, similarities in the plumage patterns of the two-banded plover (*C. falklandicus*) of South America and the double-banded plover (*C. bicinctus*) of New Zealand were suggested as evidence of a close phylogenetic relationship, and the distribution of ten plover species at the southernmost tips of southern land masses were taken to be suggestive of a common ancestor inhabiting Antarctica at a time when the continent was not covered by ice (Vaughan, 1980). Support for a Southern Hemisphere origin was provided by an analysis of mitochondrial sequence variation in 15 plovers and allies (Joseph *et al.,* 1999) that tentatively proposed South America as the ancestral home of this group. However, taxon sampling in this study was heavily biased towards species currently restricted to the Southern hemisphere whereas approximately half of the modern *Charadrius* species inhabit the Northern hemisphere (Hayman *et al.,* 1986).

Here we attempt to more rigorously address the question of a Northern or Southern origin for the genus *Charadrius.* Recently-developed methods for ancestral area reconstruction include parsimony-based, likelihood-based and Bayesian models that statistically evaluate alternative ancestral ranges at each node in a Bayesian

phylogeny taking into account phylogenetic uncertainty (Heled and Drummond, 2010; Maddison and Maddison, 2011).

To achieve this goal we estimated the most comprehensive global molecular phylogeny of the *Charadrius* plovers to date, based on sequence data from two mitochondrial (mtDNA) and four nuclear loci and constructed using a Bayesian multi-species coalescent approach (*BEAST; Heled and Drummond, 2010; Drummond *et al.,* 2012). The phylogeny included 29 currently classified *Charadrius* species (all recognised species except *C. javanicus*) as well as seven non-*Charadrius* species: four species with debated taxonomic classification, from closely-related genera (*A. frontalis, E. morinellus, T. rubricollis, T. novaeseelandiae;* Bock, 1958; Nielsen, 1975; Vaughan, 1980; Davis, 1994; Barth *et al.,* 2013); two species from the sister genus *Vanellus* (*V. miles* and *V. armatus*) and one more distantly-related outgroup species, *Pluvialis squatarola*. With this phylogeny we investigated i) the monophyly of the genus *Charadrius* and ii) their biogeographical origins, using both parsimony-based and Bayesian methods (Maddison and Maddison, 2011; Yu *et al.,* 2013). In addition we used mtDNA molecular clock data in order to provide an approximation of the age of the genus and divergence times between species to enable the identification of historical climatic conditions that coincide with major radiations in this clade.

Material and methods

Taxon sampling

Samples were collected from three individuals for a total of 36 species (including 29 *Charadrius* species; Table S1, Supplementary material). Blood samples were collected from 23 *Charadrius* species, and seven non-*Charadrius* species from wild populations following methods outlined by Székely *et al.* (2008). Toe-pad samples were collected from museum specimens at the Natural History Museum, Tring, from six further *Charadrius* species (*C. alticola, C. asiaticus, C. forbesi, C. peronii, C. placidus* and *C. obscurus*).

DNA extraction, amplification and sequencing

DNA was extracted from blood samples using an ammonium acetate precipitation method (Nicholls *et al.,* 2000; Richardson *et al.,* 2001) at the University of Sheffield. To avoid cross-contamination with blood samples, DNA extraction from museum toe-pad skin samples was conducted in a separate, dedicated pre-PCR laboratory at a different

location, Swansea University, using DNeasy Tissue Kits (Qiagen); see Bantock *et al.* (2008) for full protocol.

We amplified six loci using Polymerase Chain Reaction (PCR), two mtDNA loci: *COI* (cytochrome oxidase I) and *ND3* (NADH dehydrogenase subunit 3), and four nuclear loci: *ADH5* (alcohol dehydrogenase 5), *FIB7* (*β*-fibrinogen intron 7)*, MYO2* (myosin-2/3) and *RAG1* (recombination activating gene 1). These genes were selected based on their previous utility in species-level avian phylogenies (Chesser, 1999; Ericson *et al.,* 2003; Fain *et al.,* 2007; Fain and Houde, 2007; Johnson *et al.,* 2009). For DNA extracted from blood samples, 'universal' avian primers were used (Table S2, Supplementary material). For DNA samples extracted from toe pads, primers targeting at least one shorter region per gene were utilised to handle degradation (Table S3, Supplementary material). For the mtDNA genes *ND3* and *COI*, suitable primers were already available (Lee and Prys-Jones, 2008; Rheindt *et al.,* 2011), including a set of three primer pairs designed to amplify the *COI* gene partially ('D', 'L' and 'Q' fragments; Table S3, Supplementary material). For nuclear genes, we designed new primers using Primer3 (Rozen and Skaletsky, 2000). New primers were located in conserved regions based on alignment of full *Charadrius* sequences to improve cross-species amplification (e.g. Küpper *et al.,* 2008).

PCRs were conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler in 10 µl reaction mixes containing 3 µl Qiagen Multiplex Mix, 0.1 µM of each primer and 20-30 ng DNA. PCR conditions were as follows: 95˚C for 15 min, followed by 42 cycles of 94˚C for 30 s, T_a (primer specific annealing temperature, Tables S2 and S3, Supplementary material) for 30 s, 72˚C for 30 s, and a final extension of 72˚C for 10 min. We ran a small aliquot of the PCR products on a 1% agarose gel to ensure amplification success. PCR products were then purified using 2 µl 10x diluted ExoSAP-IT (GE Healthcare) according to the instructions of the manufacturer and subsequently sequenced. Cycle sequencing was performed by GenePool Laboratory, Edinburgh, on an ABI 3730 DNA analyser (Applied Biosystems) using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Sequence alignment and phylogenetic analyses

Alignment of forward and reverse sequences, base-calling, end-clipping and ambiguity checks were carried out in CodonCode Aligner 3.7.1 (CodonCode Corporation) using the ClustalW algorithm for alignment. For nuclear genes, heterozygote positions were coded according to the universal ambiguity code. Full sequence alignments for each gene were

produced in MEGA 5.21 (Tamura *et al.,* 2011). Best-fit nucleotide substitution models were selected based on Akaike's Information Criterion (AIC) in MrModelTest 2.3 (Nylander, 2004; Table 1). Sequence data has been deposited in the GenBank sequence database (accession numbers *ADH5 GenBank: KM001088-KM001169; MYO2 GenBank: KM001170-KM001256; COI GenBank: KO001257-KM001341; ND3 GenBank: KM001342-KM001425; FIB7 GenBank: KM001426-KM001507; RAG1: KM001508-KM001594*).

For eight cases, data was retrieved from GenBank or the Barcode of Life Database (BOLD). These sequences included: *C. hiaticula COI* (*GenBank: GU571812.1, GU571811.1, GU571331.1*; Johnsen *et al.* unpubl.), *C. falklandicus COI* (*GenBank: FJ027346.1, FJ027345.1;* Kerr *et al.* 2009), *C. leschenaultii COI* (*Genbank: DQ432845.1, GQ481569.1*; Kerr *et al.,* 2007, 2009), *C. mongolus COI* (*GenBank: GQ481572.1, GQ481571.1, GQ481570.1*; Kerr *et al.,* 2009), *C. ruficapillus ND3* (*GenBank: FR823187.1, FR823188.1, FR823189.1*; Rheindt *et al.,* 2011), *A. frontalis COI* (*BOLD: BROM379-06, BROM380-06, BROM617-07*; A.J. Baker), *E. morinellus COI* (*GenBank: DQ433492.1, GU571813.1, GU571814.1;* Kerr *et al.,* 2007; Johnsen *et al.* unpubl.) and *E. morinellus RAG1* (*GenBank: EF373182.1*; Baker *et al.,* 2007).

Phylogenetic analyses were performed in *BEAST 1.7.5 (Drummond *et al.,* 2012; Heled and Drummond, 2010) using the ICEBERG computing cluster hosted by University of Sheffield and XSEDE on the CIPRES (Cyberinfrastructure for Phylogenetic Research) gateway (Miller *et al.,* 2010). *BEAST employs a Bayesian multispecies coalescent approach and is capable of estimating divergence times, rates of gene evolution and the parameters of evolutionary models for separate gene partitions. This program co-estimates multiple gene trees embedded in a shared species tree, allowing for variation in rates of molecular evolution between loci.

Xml-files for *BEAST analyses were prepared using BEAUti 1.7.5 (BEAST package) with all sequences concatenated and each gene assigned to a separate partition. Because of their lack of recombination, mitochondrial *COI* and *ND3* genes were linked into a single partition tree. The species tree prior was set to Yule Process and the population size model set to piecewise linear and constant root. In preliminary analyses, a well-dated *E. morinellus* fossil proved uninformative for molecular clock calibration as the divergence time between *E. morinellus* and its closest relative in our dataset far pre-dates the age of the fossil (2.5 million years ago (Mya)). We therefore approximated the age of genus *Charadrius* based on a mtDNA molecular clock. Clock models were lognormal, relaxed and estimated for all genes except *ND3*. To enable estimation of divergence times, the substitution rate for *ND3* was set to 0.00233 per million years as

estimated for *Charadriiformes* based on external avian calibration points (Pereira and Baker 2006). For the remaining genes, ucld means (mean rates under the uncorrelated log-normal relaxed clock) were estimated with uniform distribution for ranges from 0 to 1 and initial values of 0.002. The Markov chains were run for 400 million generations and sampled every 15,000 generations.

Convergence was concluded from stationary distributions of MCMC (Markov chain Monte Carlo) sample traces in Tracer v1.5 (BEAST package). Summary of the posterior distribution of *BEAST trees and identification of the maximum clade credibility (MCC) tree was conducted using TreeAnnotator 1.7.5 (Drummond and Rambaut, 2007) with a burn-in value of 15% and median node heights. Four independent runs were conducted for each treatment to ensure convergence. The MCC tree was visualised in FigTree v1.4.0 (available at: *http://tree.bio.ed.ac.uk/software/figtree/*). Additionally, MCC trees were built for each gene separately based on *BEAST output in order to visualise areas of gene tree discordance.

Ancestral area reconstruction

The present-day breeding distributions of each species (data from International Union for Conservation of Nature (IUCN), 2014) were categorised into nine geographic regions. These regions were based on terrestrial zoogeographic realms and modified in line with data on phylogenetic turnover among regions in birds (Holt *et al.,* 2013) as well as the distribution of *Charadrius* plovers, such that each region is occupied by at least four modern *Charadrius* species (Fig. 1a; Table S1, Supplementary material).

For reconstruction of ancestral areas, we employed two methods that statistically evaluated alternative ancestral ranges at each node of the summarised Bayesian phylogeny, taking into account phylogenetic uncertainty. The first was a parsimony method implemented in Mesquite 2.75 (Maddison and Maddison, 2011; Ancestral States Reconstruction Package) with a step (cost) matrix model. This model accounts for the likelihood of dispersal between regions. We assumed equal transition costs for movement between adjacent realms, therefore the costs reflected the minimum number of neighbouring areas a species would have to disperse through to make the transition.

Secondly, Bayesian binary Markov chain Monte Carlo (BBM) analysis was implemented in RASP v2.1 beta (Reconstruct Ancestral States in Phylogenies; Yu *et al.,* 2010; Yu *et al.,* 2013) under a variable F81+G model for 5,000,000 generations with 10

chains sampling every 100 generations and root ancestral distribution specified as 'widespread' (global).

Results

Sequence characteristics

Properties of sequence data for each of the four nuclear and two mtDNA loci, including sequence length, nucleotide substitution models and percentage of variable positions are given in Table 1. The full sequence length of all six concatenated genes was 4295 base pairs. For DNA extracted from museum toepads the length of sequence data amplified was 1343 base pairs leading to 69% missing data in six species. No sequence data could be generated or retrieved for the following species / DNA fragments: *C. bicinctus* / *COI*, *C. placidus* / *ND3* and *RAG1*, *C. melodus* / *MYO2* and *ND3*, *C. ruficapillus* / *ADH5* and *E. morinellus / ND3*, *ADH5, FIB7* and *MYO2*, leading to a total of 5% of missing data (15% of characters, including museum specimen sequences).

Phylogeny

The maximum clade credibility tree supported a monophyletic origin for 28 of the 29 *Charadrius* species excluding only the taxon *C. modestus.* This monophyletic group also included the taxa *A. frontalis*, *T. novaeseelandiae*, *T.rubricollis* and *E. morinellus* and emerged as distinct from the *Pluvialis* and *Vanellus* genera. Two major clades were identified within the genus *Charadrius* (*CRD I* and *CRD II*; Fig. 1b). These two clades can be further categorised into seven sets of sister species (*Sets a – g*, Fig. 1b) with largely shared geographic distributions and / or morphological characteristics (Bock, 1958; Nielsen, 1975; IUCN 2014).

Clade *CRD I* included the ringed plover species – four *Charadrius* species within *Set a* (including the common ringed plover, *C. hiaticula*), and four within *Set b* (including the little ringed plover, *C. dubius*). Additionally, *Set b* included three species not presently classified as members of the genus *Charadrius*: *E. morinellus*, *T. rubricollis* and *T. novaeseelandiae*. Within *CRD II*, *Set c* included four Asian red-breasted species; *Set d* consisted of five species of the sand plover group, including *C. alexandrinus* and its allopatric sister species; *Set e* included three Oceanian species - *C. bicinctus, C. obscurus* and *A. frontalis*; *Set f* included three African species - two endangered island species (*C. thoracicus* and *C. sanctaehelenae*) and one widespread species (*C. pecuarius*); and *Set g* consisted of five American (Nearctic and Neotropical) species of the mountain and plains plover group (Vaughan, 1980). *C. ruficapillus* was identified as a member of *CRD II* but

was not assigned to a species set since no close sister species appeared within this dataset.

For the six species for which museum toepad samples were the source of DNA (*C. alticola, C. asiaticus, C. forbesi, C. peronii, C. placidus* and *C. obscurus*), sequence information was incomplete. Additionally, four *Charadrius* species were missing sequence data for one or two genes, namely *C. bicinctus, C. placidus, C. melodus* and *C. ruficapillus*, and only two gene regions were included for *E. morinellus*. Nonetheless, the phylogenetic placement of the nine *Charadrius* species with partial or missing data did not differ from expectations based on biogeographical distribution or plumage colouration (Nielsen, 1975; Hayman *et al.,* 1986). However, the position of *E. morinellus* within *CRD I* was only supported by low posterior probability (0.42).

Levels of nodal support, based on posterior probability, were above 0.9 for 18 of the 35 nodes. Overall assignment of sister species to the seven sets was highly supported (Fig. 1b, Table 2). However, lower posterior probabilities were often present at nodes within species sets. Twelve nodes across the MCC tree had posterior probabilities below 0.7 (Fig. 1b) and eight of these nodes were within *Sets b, d, g and f*, where divergence seems to have occurred over a short time scale and species-level relationships appeared polytomic. Further points of low support emerged at the two basal nodes adjoining *Set f* and the distinct species *C. ruficapillus* with neighbouring species sets, and additionally, at the two nodes linking the *Vanellus* species with *C. modestus* and the main *Charadrius* clades. These nodes corresponded to areas of gene tree discordance (Table 3; Fig. S1, Supplementary material). Firstly, the placement of *C. modestus* outside the *Charadrius* genus was supported by the gene trees of *ND3*, *COI*, *ADH5* and *FIB7*, but not by *MYO2* and *RAG1* which included the taxon as a sister taxon to the *CRD I* clade. Secondly, concerning the placement of the two *Vanellus* species (*V. miles* and *V. armatus*), four of the six gene trees supported placement outside the *Charadrius* clade. However, based on *FIB7*, both *Vanellus* species were placed as sister taxons to *CRD II,* within the *Charadrius* tree, and according to the *MYO2* gene tree, *V. miles* was included as sister taxon to *CRD II* but *V. armatus* was placed closer to the *CRD I* group.

Divergence time estimation and ancestral area reconstruction

Based on molecular clock data and the MCC species tree, the most recent common ancestor of the genus *Charadrius*, excluding *C. modestus*, existed between 79.6 and 48.6 Million years ago (95% highest posterior density (HPD), node 5; Table 2; Fig. 1b), at which time the ancestors of the *CRD I* and *CRD II* lineages diverged. Further divergence

in the direct ancestry of the modern *Charadrius* plovers began during the late Paleogene period (*CRD I*: 45.0–28.4 Mya; *CRD II*: 33.2–20.6 Mya). Within the *CRD I* clade, the Oligocene (final Paleogene epoch, 33.9–23.0 Mya) was a major period of radiation and nearly all modern species appear to have diverged from their extant sister species during this time. Within the *CRD II* clade, the earliest divergence between modern lineages took place approx. 33.2–20.6 Mya. However, in contrast to *CRD I*, the majority of modern species within *CRD II* emerged at a later stage, during the Neogene period (23–2.6 Mya).

Based on parsimony analysis, the most recent common ancestor of the *Charadrius* plovers (*CRD I* and *CRD II*, excluding *C. modestus*) originated in the Northern hemisphere, with a distribution in the Arctic, Palearctic and/or Central Asian regions (node 5, Fig. 1b). BBM analysis concurred in identifying the Northern hemisphere as the centre of origin, placing greater probability on either Central Asia or the Arctic regions and additionally including the Nearctic region within the possible ancestral range. The probability that the genus originated in the Southern hemisphere was <0.01 (parsimony) and 0.05 (BBM). Similarly, both major clades *CRD I* and *CRD II* appeared to have emerged in the Northern hemisphere. The most recent common ancestor (MRCA) of Clade *CRD I* (node 6, Fig. 1) was likely to have occupied a similar Northern hemisphere range as the *Charadrius* MRCA, whereas the *CRD II* clade was more likely to have emerged in Central Asia or the Palearctic than in Arctic or Nearctic regions (node 16, Fig. 1).

During subsequent evolutionary radiation, ancestral *Charadrius* plovers moved southwards and colonised Africa three times (within *Sets b, d* and *f*) and the Oriental - Oceanian regions at least three times (within *Sets b, d and e* as well as ancestral *C. ruficapillus*). Consistently, the two extant species (*C. alticola* and *C. falklandicus*) that breed solely within the Neotropical region diverged within *Set g* from North American ancestors during the Neogene period.

Table 1: Characteristics of mitochondrial and nuclear loci used in the phylogenetic analysis of *Charadrius* and seven allied species.

Gene	Length (bp)		Nucleotide substitution model	Mean pairwise identity (%)
	Fresh samples	Museum samples (% of full sequence)		
ND3[a]	401	200 (50)	HKY + I + G	88.6
COI[a]	626	429 (69)	GTR + I + G	89.3
ADH5[b]	829	150 (18)	GTR + G	95.5
FIB7[b]	840	138 (16)	GTR + G	95.2
MYO2[b]	688	209 (30)	HKY + G	96.4
RAG1[b]	911	217 (24)	HKY + I + G	98.2

[a] mitochondrial loci; [b] nuclear loci

Table 2: Posterior probabilities (PP) and 95% highest posterior density (HPD) intervals on divergence time estimates for nodes in the maximum clade credibility tree. See nodes in Fig. 1.

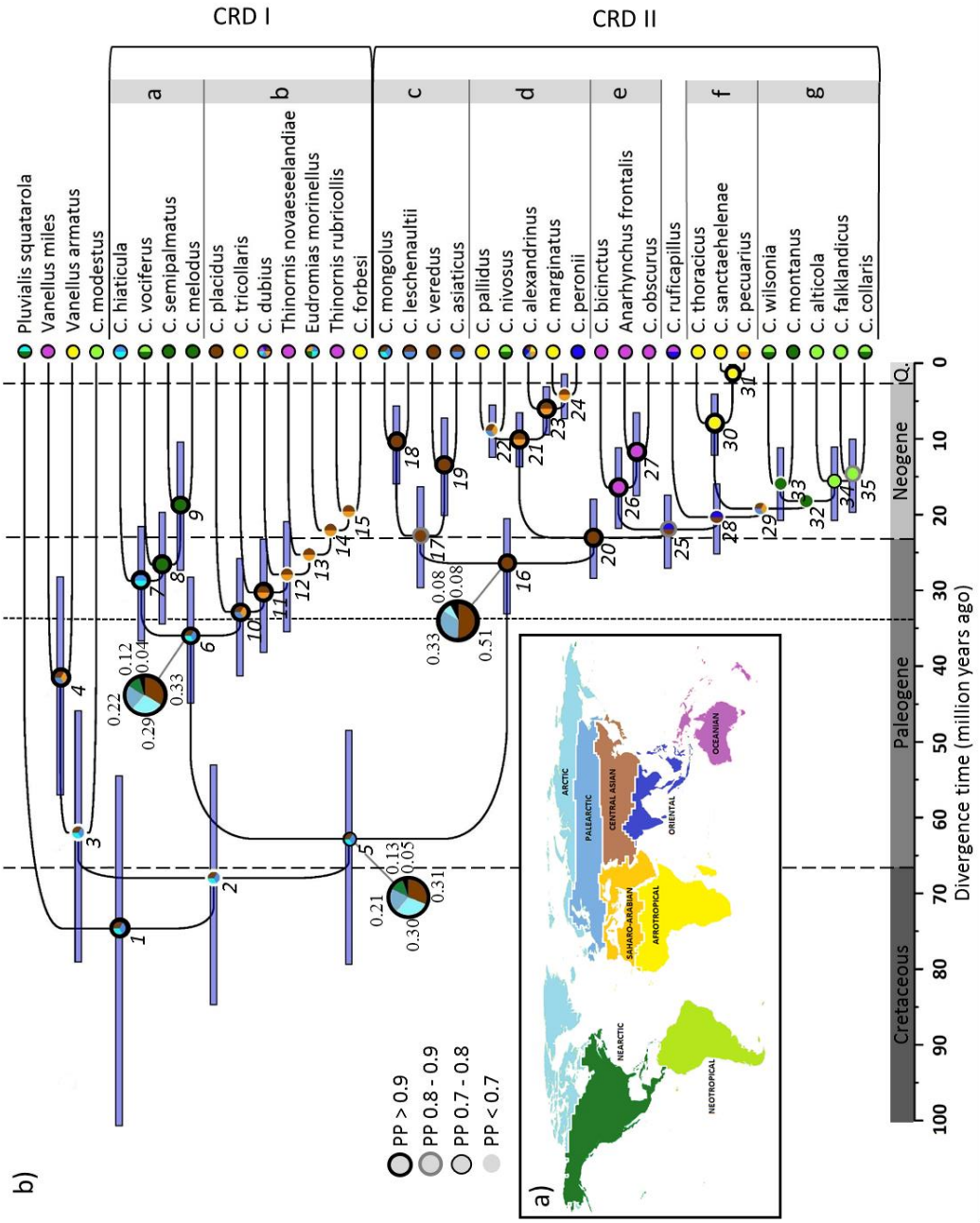
Node	PP	95% HPD		Node	PP	95% HPD	
		Lower	Upper			Lower	Upper
1	1.00	54.6	100.9	19	1.00	7.3	20.2
2	0.65	53.2	84.9	20	0.97	18.0	28.5
3	0.62	46.0	79.3	21	1.00	6.6	13.8
4	1.00	28.3	57.2	22	0.55	5.6	12.5
5	0.72	48.6	79.6	23	0.93	3.2	9.5
6	1.00	28.4	45.0	24	0.58	1.5	7.4
7	1.00	21.7	36.8	25	0.84	17.5	27.2
8	0.94	19.8	34.5	26	0.99	11.2	21.9
9	0.98	10.5	27.5	27	0.99	6.6	17.6
10	0.92	25.9	41.4	28	0.64	16.1	25.3
11	0.94	23.3	38.3	29	0.34	15.1	23.8
12	0.65	21.0	35.6	30	1.00	4.1	17.6
13	0.48	18.5	32.8	31	1.00	0.2	2.6
14	0.42	13.8	30.4	32	0.47	14.1	23.0
15	0.36	11.4	28.0	33	0.51	11.3	20.8
16	1.00	20.6	33.2	34	0.71	11.2	20.9
17	0.83	16.4	29.7	35	0.89	10.1	19.8
18	1.00	5.7	16.0				

Table 3: Gene tree to maximum clade credibility tree comparisons.

Species set (number of species)	Fully supportive gene trees	Number of species excluded from sets					
		COI	*ND3*	*ADH5*	*FIB7*	*MYO2*	*RAG1*
a (4)	4	*	*	1	1	*	*
b (7)	0	2	2	2	1	3	1
c (4)	3	1	*	*	2	*	2
*d (*5)	5	*	*	*	*	1	*
e (3)	4	*	*	*	1	*	1
f (3)	5	*	*	*	1	*	*
g (5)	1	1	1	1	2	*	1
C. modestus (1)	4	*	*	*	*	1	1
Vanellus (2)	4	*	*	*	2	1	*

* indicates fully supported species sets. See Fig. S2 (Supplementary material) for gene trees.

Figure 1: a) Biogeographical regions (revised from Holt *et al.* 2013) used to define current breeding distributions for each species. b) The maximum clade credibility tree for 29 *Charadrius* and seven outgroup species. Results of parsimony ancestral area analysis are shown for all nodes (pie chart colours by region) and BBM results with probability distributions are added for the three basal *Charadrius* nodes (larger pie charts). Species sets within clades *CRD I* and *CRD II* are labelled a – g. Broken lines indicate transitions between geological periods in relation to molecular clock divergence times (Q: Quaternary period). The dotted line indicates the time of the Eocene-Oligocene extinction event. Node circle outlines indicate posterior probabilities. Details for each node are given in Table 2.



Discussion

Phylogenetics and taxonomy

Our global molecular phylogeny of the *Charadrius* plovers moves forward from previous work in untangling the controversial evolutionary history of the genus. Firstly, our results were consistent with the more traditional phylogenetic hypothesis that nearly all *Charadrius* species form a largely monophyletic clade distinct from the *Vanellus* genus. Although the placement of one species, *C. modestus,* was still not fully resolved, the majority of the evidence suggested that *C. modestus* represented a sister lineage to *Charadrius* and *Vanellus*. By contrast, the recent classification of the wrybill (*A. frontalis*) and the two *Thinornis* species, *T. rubricollis* and *T. novaeseelandiae*, as members of the genus as suggested by Barth *et al.* (2013), was supported by our more extensive and more complete data set. Lastly, ancestral area reconstruction and molecular clock data indicated that the MRCA of the genus evolved in the Northern hemisphere and diversification in the lineages leading to modern *Charadrius* species coincided with dispersal southwards, during the late Paleogene (Oligocene) and Neogene periods.

Our results were based on a Bayesian multi-species coalescent approach, including 95% complete sequence data from four nuclear and two mtDNA loci, and including all but one *Charadrius* species, in order to recover the most likely species tree, taking into account levels of phylogenetic uncertainty and discordance across gene trees (Brito and Edwards, 2009; Corl and Ellegren, 2013). We identified seven sets of sister species within the genus and these were strongly coherent with geographic distributions and morphological characters (e.g. plumage colouration; Bock, 1958; Nielsen, 1975; IUCN 2014). Of particular importance for conservation biology was the result that the Kittlitz's plover (*C. pecuarius*) of Africa was the closest widespread sister species of the 'critically endangered' St Helena plover (*C. sanctaehelenae*) and 'vulnerable' Madagascar plover (*C. thoracicus*).

Whilst our results often matched those of Barth *et al.*'s (2013) partial dataset, there was disagreement in three areas: firstly, we provided new evidence on the positioning of one Nearctic-breeding species, *C. montanus*, as a sister species to *C. wilsonia* (a Nearctic/ Neotropical species) whereas Barth *et al.* (2013) suggested that *C. wilsonia* shared more recent ancestry with the Afrotropical *C. pecuarius* and Oceanian *C. ruficapillus*; secondly, there was disagreement concerning the order of divergence between species sets in *CRD II*; and thirdly, at the genus level, and of significance to our understanding of the evolutionary history of the genus, our results indicated that the

genus *Vanellus* diverged from the MRCA of the genus *Charadrius* prior to the first radiation that led to the formation of the modern species. All three points of difference occurred in areas of the *Charadrius* phylogeny where nodes in our phylogeny were supported by lower posterior probabilities and gene trees were discordant. In particular, we noted the contrasting signal provided by the *FIB7* (and *MYO2*) gene for recovering the phylogenetic position of the *Vanellus* species. *FIB7* sequence data were also included in analyses by Barth *et al.* (2013) and we suggest that this may have led to their rather surprising placement of the genus *Vanellus* within the *Charadrius* clade. Additionally, our placement of *C. modestus* outside *Charadrius* did not receive full support across all gene trees with *C. modestus* appearing closer to clade *CRD I* based on data from two out of six genes (*MYO2* and *RAG1*).

Discordance between gene trees and resulting phylogenies may be caused by different processes. Already identified as a major issue in recovering phylogenetic histories at deeper levels of the *Charadriiform* tree (Baker *et al.,* 2012; Corl and Ellegren, 2013), gene tree discordance is common when lineages have emerged following periods of rapid radiation. Such radiation commonly leads to incomplete lineage sorting, making it difficult to disentangle true orders of divergence in species trees (Degnan and Rosenberg, 2006; Knowles and Chan, 2008; Chung and Ané, 2011; Corl and Ellegren, 2013). Speciation is often a gradual, extended process rather than a single point event and gene flow commonly occurs after initial divergence (Avise and Walker, 1998). Additionally, hybridisation or introgression between species can enable gene flow even after species divergence (Kubatko, 2009).

Technical advances in sequencing methodology and further reduction of sequencing and assembly costs will soon help to determine with greater confidence the evolutionary relationships between all species. These advances, facilitating multiple-locus and soon genome-wide sequence analyses, will enable the use of a newly emerging phylogenomic approach to infer evolutionary history (Delsuc *et al.,* 2005) and may perhaps provide greater resolution in uncertain areas of the *Charadrius* phylogeny in future.

Ancestral origins and biogeography

We estimated the divergence of ancestral *Charadrius* plovers from the genera *Pluvialis* and *Vanellus* to have occurred between 100.9–54.6 Mya and 84.9–53.2 Mya (95% HPD) respectively. These results were consistent with a higher level molecular shorebird phylogeny which estimated the MRCA of *Pluvialis* and *Charadrius* at 60–78.8

Mya and of *Vanellus* and *Charadrius* at 52.9–71.5 Mya (Baker *et al.* 2007). However, the estimated age of the MRCA of the genus *Charadrius* (79.6–48.6 Mya; *CRD I* and *II*) based on our results was older than previous estimates (46–31 Mya; Barth *et al.* 2013). Confidence intervals for dating were broad as estimates were produced using a mtDNA molecular clock based on mutation rates at deeper nodes in the avian tree of life (Pereira and Baker, 2006). To enhance estimations and recover more precisely the age of the genus, it would be necessary to include fossil calibration points. Unfortunately, there are no reliable and informative fossils currently available for the small plovers and previous attempts to utilise fossil data in calibrating a *Charadriiform* phylogeny (Baker *et al.,* 2007) have been criticised for including incorrectly classified fossils (Mayr, 2011; Parham *et al.,* 2012). In their review of 'best practices for justifying fossil calibration', Parham *et al.* (2012) reported only one fossil of potential use in dating plover phylogenies based on their checklist of calibration criteria, namely a fossil of *E. morinellus* found in Poland from the late Pliocene (MN16; 2.5 Mya; Jánossy, 1974). However, in preliminary analyses, this single fossil proved uninformative as the divergence time between *E. morinellus* and its closest relative in our dataset far pre-dates the age of the fossil.

The estimated age of the *Charadrius* MRCA greatly pre-dates speciation events within either clade *CRD I* or *CRD II*. Speciation processes were probably ongoing during the Paleogene period but mass extinction events may have had a major impact on the past phylogenetic diversity of the genus. Specifically, our results suggested that the genus emerged from a single ancestral survivor of the Cretaceous-Paleogene extinction event (66 Mya). Diversification is likely to have taken place subsequently during the early Paleogene, yet very few lineages may have survived the Eocene-Oligocene extinction event ("Grande Coupure", 34 Mya) and later radiation within these lineages gave rise to the modern *Charadrius* species.

CRD I and *CRD II* radiations occurred at different times. Within *CRD I*, most modern species diverged from their closest sister species during the Oligocene, whereas within clade *CRD II*, speciation leading to the modern species mostly occurred later, during the Miocene epoch. There may be two explanations for these differences: firstly, speciation may have taken place but rates of extinction may have been higher within *CRD I* than *CRD II*; secondly, population connectivity may have been higher for species in *CRD I* than *CRD II,* preventing genetic isolation and speciation in *CRD I* species but not in *CRD II* species. The extant members of each clade occupy broadly similar geographic areas, therefore it is difficult to determine which hypothesis is most likely. We do note,

however, that a number of *CRD II* species are characterised by very high gene flow (Küpper *et al.* 2009, 2012), therefore it is unlikely that differences in population connectivity are the sole reason for differences in radiation times. The emergence of fossil evidence, and further population genetic studies, may help to clarify the reasons behind these radiation differences.

Our results overwhelmingly supported a Northern hemisphere rather than Southern hemisphere origin for the genus. Parsimony and BBM analyses were consistent in their support for the Northern hemispheric origin, although the exact zoogeographic region of origin could not be determined. The modern *CRD I* species were largely distributed within the Northern hemisphere (seven Northern, four Southern) whereas *CRD II* species breed largely in the Southern hemisphere (eight Northern, 13 Southern). Despite their differing distributions both clades were identified as originating in the Northern hemisphere. The most likely distribution of the ancestral *CRD II* species was in Central Asia, in contrast to the broader Northern hemisphere (Arctic, Palearctic, Central Asian and Nearctic) origin of *CRD I*, suggesting that radiation within *CRD II* began at a lower latitude in the Northern hemisphere than for *CRD II*. We reject the Southern hemisphere/Antarctic origin theory of Vaughan (1980) for two additional reasons: firstly, we found no support for *C. falklandicus* and *C. bicinctus* as sister species in South America and New Zealand; secondly, the estimated age of the genus at approximately 63 Mya (95% HPD: 48.6–79.6 Mya), places the emergence of these plovers at a time when Antarctica had already been separate from Africa and Australia for approx. 100 and 20 million years respectively (Boger, 2011) and were separated by a several thousand kilometre stretch of ocean. Instead, we suggest that these continents were colonised following southward dispersal by Northern hemisphere populations. The similarity in plumage patterns between *C. falklandicus* and *C. bicinctus* is therefore likely to be the result of convergent evolution.

The Northern hemisphere, Paleogene origins identified for the *Charadrius* plovers in this study coincided with large-scale biogeographical patterns reported across a range of avian and mammalian taxa (Zachos *et al.,* 2001; Hunt, 2004; Maguire and Stigall, 2008; Schweizer *et al.,* 2011). Studies of the fossil record have identified the Palearctic in particular as an important region in the diversification of the very first bird species, being occupied by members of all modern orders of birds during the early Paleogene (65–55 Mya; Blondel and Mourer-Chauviré, 1998). Furthermore, the order *Charadriiformes* is considered among the earliest bird lineages to have survived the Cretaceous-Paleogene extinction event (Feduccia, 1995; Wesołowski, 2004; Baker *et al.,*

2007). Our results suggested that following emergence of the first ancestral *Charadrius* species, much subsequent diversification among ancestral populations took place during the Oligocene (34–23 Mya) and Miocene (23–5 Mya) periods at times of major climatic oscillation and global cooling (Feduccia, 1995; Blondel and Mourer-Chauviré, 1998; Zachos *et al.,* 2001). Dramatic changes in climate over these time periods led to rapid evolutionary radiation among many taxa, and species often dispersed from higher latitudes towards lower latitudes, 'tracking' more preferable temperatures and habitats (Maguire and Stigall, 2008; Schweizer *et al.,* 2011). We speculate that the processes of global cooling and subsequent climatic oscillation during the Miocene period were therefore likely to have been important in the colonisation of the Southern hemisphere by the plovers, driving speciation within the genus *Charadrius*. Furthermore, this ability to 'track' the climate and disperse to new habitats may be highly important in the viability of threatened shorebird populations in the face of future climate change.

Conclusions

Our new global phylogeny of the genus *Charadrius* provides much needed information on the evolutionary history of a diverse group of shorebirds. This group is emerging as ideal for studying the evolution of a range of phenotypic traits including breeding systems, migration strategies and plumage (Owens *et al.,* 1995; van de Kam *et al.,* 2004; Argüelles-Ticó, 2011). To date, studies on the genus *Charadrius* have focussed on microevolutionary patterns, investigating just one or a few closely related species (e.g. Székely *et al.,* 2004b; Vincze *et al.,* 2013). This new phylogeny of the genus *Charadrius* provides a robust framework enabling larger scale investigations on how evolutionary changes emerge in complex traits at the species level. Whilst resolving many areas of taxonomic controversy, our phylogeny also highlights key points of gene tree discordance. Future phylogenetic studies should aim to resolve these points by examining more molecular markers, making use of advancements in sequencing technologies.

Acknowledgements

Samples were generously provided by Bernard Amakobe, Matilde Alfaro, Araceli Argüelles-Ticó, Marcela Beltrán, Fiona Burns, Stephen J. Dinsmore, Jim de Fouw, Balazs Kaholek, Sylvester Karimi, Terje Lislevand, Gabriel Garcia-Peña, Susan Haig, Craig Millar, Christine Ochieng, Ian Owens, Jorge Parra, Paul D. B. Skrade, James St Clair, Bela Tokody, Pavel Tomkovich, Mike Weston and Sama Zefania. We also thank Mark Adams, Robert

Prys-Jones and Douglas Russell for sample collection from museum specimens at the Natural History Museum, Tring. Sequence analysis was supported by NERC-Biomolecular Analysis Facility at the University of Edinburgh and at the University of Sheffield. Gavin Thomas provided statistical advice on phylogenetic analyses and Allan Baker kindly commented an earlier draft of this manuscript. This study was funded by the Natural Environment Research Council as part of the PhD research of NdR. CK was supported by a Marie Curie IEF postdoctoral fellowship. Permits for fieldwork and collection of samples were provided by the national agencies in each country.

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Supplementary Material

Supplementary Table 1. List of species included in global phylogenetic analyses, ordered by breeding distribution. Biogeographic region refers to current breeding distributions. *Samples collected from museum specimens (including museum accession numbers; Natural History Museum, Tring).

Species	Common name	Biogeographic region	Sampling location	Collectors (museum accession numbers)
C. melodus	Piping Plover	Nearctic	USA	S. Haig
C. montanus	Mountain Plover	Nearctic	USA	S.J. Dinsmore, P.D.B. Skrade
C. semipalmatu	Semipalmated Plover	Nearctic	Canada	S. Haig
C. collaris	Collared Plover	Nearctic, Neotropical	Uruguay	M. Alfaro
C. nivosus	Snowy Plover	Nearctic, Neotropical	Mexico	C. Küpper
C. vociferous	Killdeer	Nearctic, Neotropical	Mexico	S. Haig
C. wilsonia	Wilson's Plover	Nearctic, Neotropical	Mexico	C. Küpper
*C. alticola**	Puna Plover	Neotropical	Bolivia/ Peru	A. Morrison, P.O. Simmons (1946.49.186; 1946.49.187; 1902. 3.13.1701)
C. falklandicus	Two-banded Plover	Neotropical	Argentina	G. Garcia-Peña
C. modestus	Rufous-chested Dotterel	Neotropical	Falkland Islands	J. St Clair
C. hiaticula	Ringed Plover	Arctic, Palearctic	Norway	T. Lislevand
C. alexandrinus	Kentish Plover	Palearctic, Saharo-Arabian,	Cape Verde	A. Argüelles-Ticó, T. Székely
C. dubius	Little Ringed Plover	Arctic, Palearctic, Saharo-Arabian,	China	T Székely
*C. asiaticus**	Caspian Plover	Palearctic, Central Asian	Malawi/ Tanzania	C.W. Benson, R.H.W. Pakenham, N.R.F. Couchman (1946.5.144; 1947.5.5; 1945.48.15)
C. leschenaultii	Greater Sandplover	Palearctic, Central Asian	Oman	J. de Fouw
*C. placidus**	Long-billed Plover	Central Asian	Tibet/ Nepal/ China	F. Ludlow, H. King Robinson, G. Forrest (1921.7.12.111; 1933.11.13.108; 1955.1.925)
C. mongolus	Lesser Sandplover	Arctic, Palearctic, Central Asian	Oman	J. de Fouw

*C. peronii**	Malaysian Plover	Oriental	Cambodia/ Malaysia	A. Morrison, W.J.F. Williamson (1955.1.925; 1949.34.48; 1936.4.12.175)
C. veredus	Oriental Plover	Central Asian	New Zealand	C. Millar
*C. forbesi**	Forbes' Plover	Afrotropical	Nigeria/ Sierra Leone	W. Serle (1946.40.7; 1951.34.94; 1966.16.32)
C. marginatus	White-fronted Plover	Afrotropical	Namibia	T. Székely
C. pallidus	Chestnut-banded Plover	Afrotropical	Kenya	N. dos Remedios
C. pecuarius	Kittlitz's Plover	Afrotropical	Kenya	N. dos Remedios
C. sanctaehelen	St Helena Plover	Afrotropical	St Helena	F. Burns
C. thoracicus	Madagascar Plover	Afrotropical	Madagascar	J. Parra
C. tricollaris	Three banded Plover	Afrotropical	Madagascar	J. Parra
C. bicinctus	Double-banded Plover	Oceanian	New Zealand	C. Millar
*C. obscurus**	New Zealand Dotterel	Oceanian	New Zealand	W.R. Wason, J. Haast, W. Saunders (1926.10.10.13; 96.7.1.331; 96.7.1.332)
C. ruficapillus	Red-capped Plover	Oriental, Oceanian	Australia	M. Weston
Anarhynchus frontalis	Wrybill	Oceanian	New Zealand	C. Millar
Pluvialis squatarola	Grey Plover	Nearctic, Arctic	Oman	J. de Fouw
Thinornis novaeseeland	Shore Plover	Oceanian	New Zealand	C. Millar
Thinornis rubricollis	Hooded Plover	Oceanian	Australia	M. Weston
Vanellus armatus	Blacksmith Plover	Afrotropical	Kenya	N. dos Remedios
Vanellus miles	Masked Lapwing	Oceanian	New Zealand	C. Millar

Supplementary Table 2. Details of primers used for PCR amplification of full length sequence fragments for two mitochondrial and four nuclear loci.

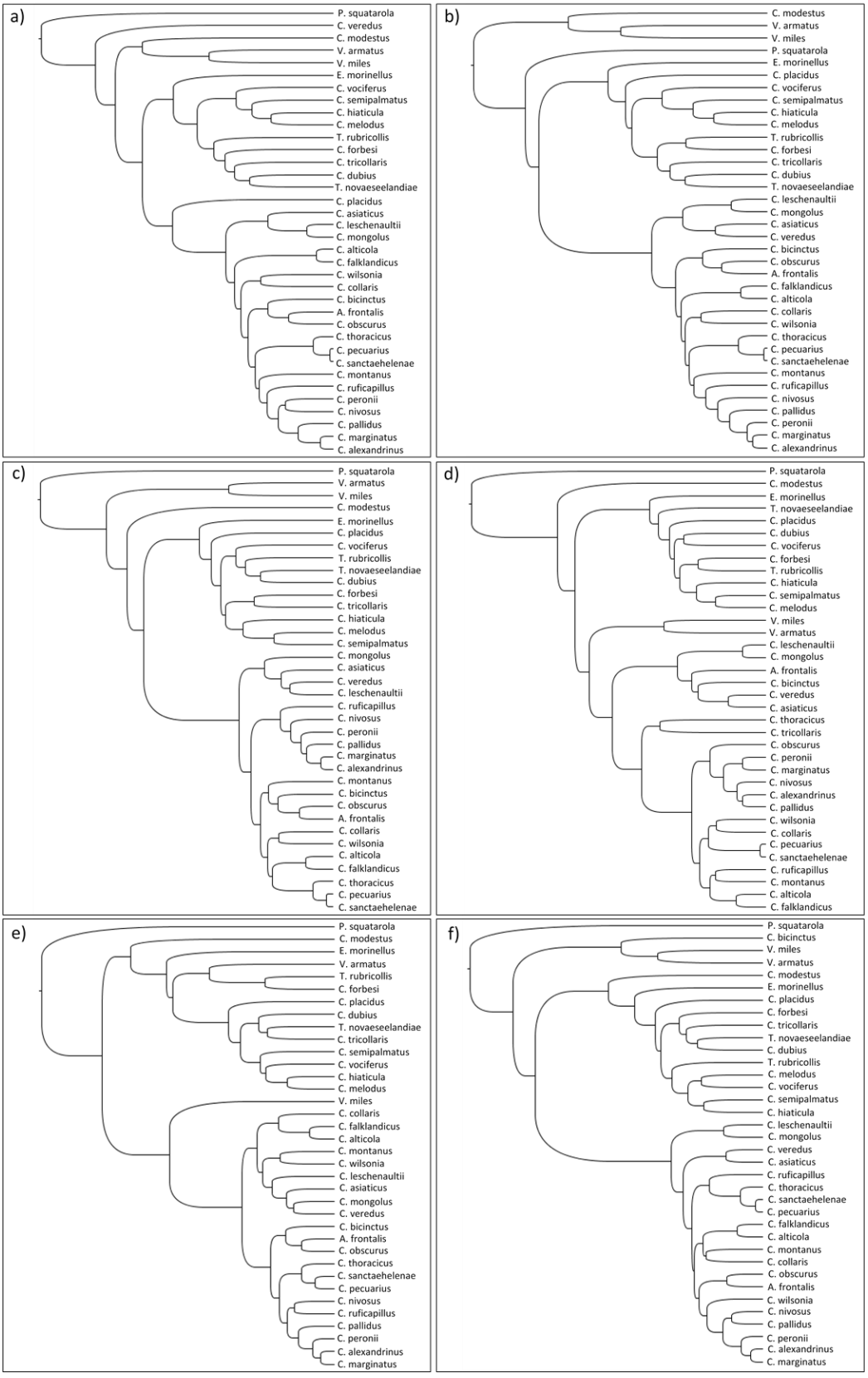
Gene	Primer	Forward/ reverse	Primer sequence 5' – 3'	T_a (°c)	Reference
COI	Bird F1	F	TTCTCCAACCACAAAGACATTGGCAC	58	Hebert *et al.* 2004
	Bird R1	R	ACGTGGGAGATAATTCCAAATCCTG		
	Bird R2*	R	ACTACATGTGAGATGATTCCGAATCCA G		
ND3	L10755	F	GACTTCCAATCTTTAAAATCTGG	62	Chesser 1999
	H11151	R	GATTTGTTGAGCCGAAATCAAC		
FIB7	FIB-BI7U	F	GGAGAAAACAGGACAATGACAATTCAC	58	Prychitko & Moore 1997
	FIB-BI7L	R	TCCCCAGTAGTATCTGCCATTAGGGTT		
MYO2	Myo-2	F	GCCACCAAGCACAAGATCCC	62	Slade *et al.* 1993
	Myo-3	R	CGGAAGAGCTCCAGGGCCTT		
ADH5	ADH5	F	TCTGTTGTCATGGGCTGCAAG	64.5	Fain *et al.* 2007
	ADH6	R	TCCAAAGACGGACCCTTTCCAG		
RAG-1	R17	F	CCCTCCTGCTGGTATCCTTGCTT	64.5	Groth & Barrowclough 1999
	R22	R	GAATGTTCTCAGGATGCCTCCCAT		

*Utilised for *C. collaris*, *C. falklandicus*, *C. leshenaultii* and *C. mongolus*.

Supplementary Table 3. Details of primers targeting shorter fragments of sequence that were utilised for PCR amplification of DNA extracted from museum specimens.

Gene	Primer	Forward/ reverse	Primer sequence 5' – 3'	T_a (°c)	Reference
COI	(D) SNIPE3	F	GCCATYAAYTTYATCACAACTGC	57	Lee & Prys-Jones 2008
	(D) SNIPE4	R	AATGTWGTGTTTAGGTTTCGRTCTG		
	(L) SNIPE6	F	GACATAGCATTTCCTCGCATA	57	
	(L) SNIPE2	R	TGGGGGTTTTATGTTRATRG		
	(Q) SNIPE8	R	TTCCAGCTCCTGCTTCTACTG	57	P.L.M. Lee pers. comm.
	(Q) SNIPE9	F	ATCGTCACTGCCCATGCTTT		
ND3	N01short	F	CCGAAATCAACTGTCTTTGTT	57	Rheindt *et al.* 2011
	N01short	R	CTTCCTCAGTAGCAATCCTATTC		
FIB7	dRS05	F	CTCCCAAAGAGATGCAGCTAA	60	This study
		R	AGCAAGCAGATCAACAGAGTACA		
MYO2	dRS06	F	AGGAATAGAGCCCAGTTCTTCTG	60	This study
		R	TCAGAAATGAACTGTGAGGAAGG		
ADH5	dRS32	F	CATGGTGTGGACTACTCCTTTG	60	This study
		R	TGTACTTCTGTCCCTGCATCTC		
RAG-1	dRS41	F	AAATGAACACAGACAAGCAGATG	60	This study
		R	AACCTGGTAGGAGGGCTTTC		

Supplementary Figure 1. *BEAST maximum clade credibility trees based on sequence data from six genes: a) *COI**, b) *ND3**, c) *ADH5*, d) *FIB7*, e) *MYO2* and f) *RAG-1*. *Mitochondrial gene.



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6 Evolutionary history and conservation genetics of African *Charadrius* plovers

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Abstract

The small plovers, genus *Charadrius,* are widespread shorebirds, breeding across every continent except Antarctica. However, many species are currently in decline. Previously, we presented the first global molecular phylogeny of the genus and identified Africa as one of the locations of most recent speciation (Chapter 5). This continent is home to three species of particular conservation interest: the 'critically endangered' St Helena plover (*C. sanctaehelenae*), 'vulnerable' Madagascar plover (*C. thoracicus*) and 'near-threatened' chestnut-banded plover (*C. pallidus*). We here further investigate patterns of evolutionary diversification by conducting phylogenetic analyses at both the interspecific and intraspecific levels based on nuclear and mitochondrial sequence data. We include all *Charadrius* species that breed within Africa, including multiple populations of four species (*C. pallidus*, *C. pecuarius*, *C. marginatus* and *C. tricollaris*) with widespread or disparate distributions across the continent. Our results provide support for the species-level relationships we presented previously and, additionally, identify genetic structure consistent with subspecies status among African populations of the white-fronted plover (*C. marginatus*), three-banded plover (*C. tricollaris*) and chestnut-banded plover (*C. pallidus*), but not the Kittlitz's plover, with implications for the influence of breeding systems on population-level genetic differentation. Both species- and population-level analyses highlight islands, in particular Madagascar, as important locations in the genetic diversification of the small plovers.

Author contributions

NdR: sample acquisition, DNA extraction, sequence analysis, phylogenetic and statistical analyses, manuscript preparation
CK: sample acquisition, phylogenetic advice, manuscript improvement
PLML – sample acquisition (museum specimens), DNA extraction (museum specimens), phylogenetic advice, manuscript improvement
TB: manuscript improvement
TS: sample acquisition, manuscript improvement

Introduction

In recent years, phylogenetic data have increasingly been incorporated into conservation strategies, for example, focussing conservation efforts by defining taxonomic units for protection (Moritz 1994; Petit *et al.* 1998; Frankham 2010); enabling the comparison of current and historical distribution patterns and evolutionary processes (Harvey 1996; Fisher & Owens 2004); predicting species' future responses to extrinsic threats such as habitat loss or climate change (Thomas *et al.* 2006; Murray *et al.* 2011); and providing a statistical framework for investigating the impact of anthropogenic threats on biodiversity (Blumstein *et al.* 2005; Macqueen *et al.* 2012). Investigating the evolutionary relationships between endangered species and their more widespread sister-species using a phylogenetic approach is the first step towards understanding the intrinsic traits that may lead some species to thrive whereas others struggle to survive (Amano & Yamaura 2007; Collen *et al.* 2011; Murray *et al.* 2011). Additionally, with such knowledge, conservationists are better equipped to make well-informed decisions on the most appropriate course of action for the prevention of future population decline, both for currently endangered species, and sister species that may be at risk in future (Amano *et al.* 2010).

The small plovers of genus *Charadrius* are cosmopolitan in distribution, breeding across every continent except Antarctica (Piersma and Wiersma 1996). However, many species are currently in decline as a result of the degradation and loss of wetland habitats, often caused in part by anthropogenic activities (Simmons *et al.* 2007; Yasué *et al.* 2007; Cohen *et al.* 2009). Africa is a location of particular interest as it is home to more species than any other continent - nine out of 30 members of genus *Charadrius* breed here, including several species of conservation concern. In Chapter 5, we presented the first global molecular phylogeny of the genus, and identified Africa as one of the locations of most recent diversification. We found that the African species do not belong to just one lineage, as would be expected from a single ancestral colonisation event, but instead appear in three distinct species groups (Subsets A – C, Fig. 1), suggesting that colonisation probably occurred at least three times in the evolutionary history of the modern African *Charadrius* plovers.

We now extend this phylogenetic work, based on data from single populations, by analysing mitochondrial and nuclear sequence data from multiple populations of four African species (*C. pallidus*, *C. pecuarius*, *C. marginatus* and *C. tricollaris*) with widespread or disparate distributions across the continent, as well as their endangered sister species, characterised by small population sizes and restricted ranges. We aim to

conduct an investigation into the evolutionary history of the African plover species with a view to identifying the evolutionary processes that may have led to current geographic distributions, and with the potential to inform conservation strategies in the future. For example, determining the evolutionary history of endangered species and their closest sister species, in relation to past changes in climate or habitat, can highlight particular populations which may be most at risk of decline in the face of future environmental change. Additionally, determining effective population size (Ne) over time and investigating the occurrence and timing of population bottlenecks can provide information on possible inbreeding depression and the likelihood of future population stability.

Of particular conservation interest is the St Helena plover (*C. sanctaehelenae*) a 'critically endangered' species (IUCN 2013) endemic to the island of St Helena. Recent estimates have placed its population size between 200 and 400 individuals (Delany *et al.* 2009; Burns 2011), with factors such as human-introduced predators and a decline in sheep-grazed farmland responsible for declining numbers (McCulloch 2009). However, thanks to conservation efforts the population is now on the increase (IUCN 2013). The 'vulnerable' Black-banded (Madagascar) plover (*C. thoracicus*; population size ~3,100 individuals; Long *et al.* 2008; IUCN 2012) is a close phylogenetic relative (*Subset f*, Chapter 5) and another endemic species of conservation concern due to its declining numbers and restricted range. Interestingly, both species emerged from the same ancestral lineage as the extremely widespread African species, the Kittlitz's plover (*C. pecuarius;* >130,000 individuals; Delany *et al.* 2009; Subset A, Fig 1), and it is currently not known why such close relatives as these may differ so greatly in distribution and population size. With data from five populations of the Kittlitz's plover (four on mainland Africa and one on Madagascar), we aim to investigate population-level phylogenetic structure within this widely distributed species, and to assess its relationship to the two more endangered species.

The 'near threatened' chestnut-banded plover (*C. pallidus*) is another species of conservation interest due to its dependence on a small number of breeding sites, several of which are under threat from pollution, siltation and water abstraction (Delany *et al.* 2009; IUCN 2013). Furthermore, during the non-breeding season, 85% of the population may inhabit just three foraging sites: Sandwich harbour and Walvis Bay, Namibia and Lake Natron, Kenya (Simmons *et al.* 2007). The species is currently described as two geographically discrete subspecies, *C. pallidus pallidus* of southern Africa and *C. pallidus venustus* of Kenya and Tanzania (Hockey *et al.* 2005; Delany *et al.* 2009). Using sequence

data from one population of the former and two populations of the latter, we aim to investigate levels of genetic divergence and the suitability of subspecies classification. The chestnut-banded plover is included within Subset B (*Subset d,* Chapter 5; Fig. 1), a group that includes the Kentish plover (*C. alexandrinus*) an extremely widespread species across Eurasia and northern Africa, and the white-fronted plover (*C. marginatus*) a widespread African species, as well as the Malaysian plover (*C. peronii*). Previous studies have placed these species within a superspecies that also includes the snowy plover (*C. nivosus*) of North and South America (until recently classified as conspecific with the Kentish plover; Küpper *et al.* 2009) and the red-capped plover (*C. ruficapillus*; Hayman *et al.* 1986; Sibley & Monroe 1990). We therefore additionally include these non-African breeding species in this study to re-assess species-level relationships within this clade, and to investigate the phylogenetic relatedness of populations.

The final set of *Charadrius* plovers breeding in Africa includes the little ringed plover (*C. dubius*), the three-banded plover (*C. tricollaris*) and Forbes's plover (*C. forbesi;* Subset C, Fig. 1; *Subset b,* Chapter 5). All notable for the brightly coloured yellow or red rings around their eyes, the latter two species appear most morphologically similar and have largely non-overlapping ranges: the three-banded plover being widespread across Africa and Madagascar with the Forbes's plover replacing it in western Africa, suggesting relatively recent allopatric speciation. However, our global study placed the divergence times between these species at approximately 15 Mya, much further back in time than for the other two African species sets. We here investigate the evolutionary relationships within this group, including multiple populations of the three-banded plover, on mainland Africa and Madagascar.

Material and methods

Taxon sampling

Samples were collected from all nine species of small plover, genus *Charadrius*, with breeding populations in Africa, as well as three closely-related non-African species. Samples were collected from three individuals per population. For ten species (19 wild populations, Table 1), 25 - 50 µl of blood was taken and stored in Queen's Lysis Buffer (Seutin *et al.* 1991) or 1 ml of 95% ethanol. For the remaining two species, *C. forbesi* and

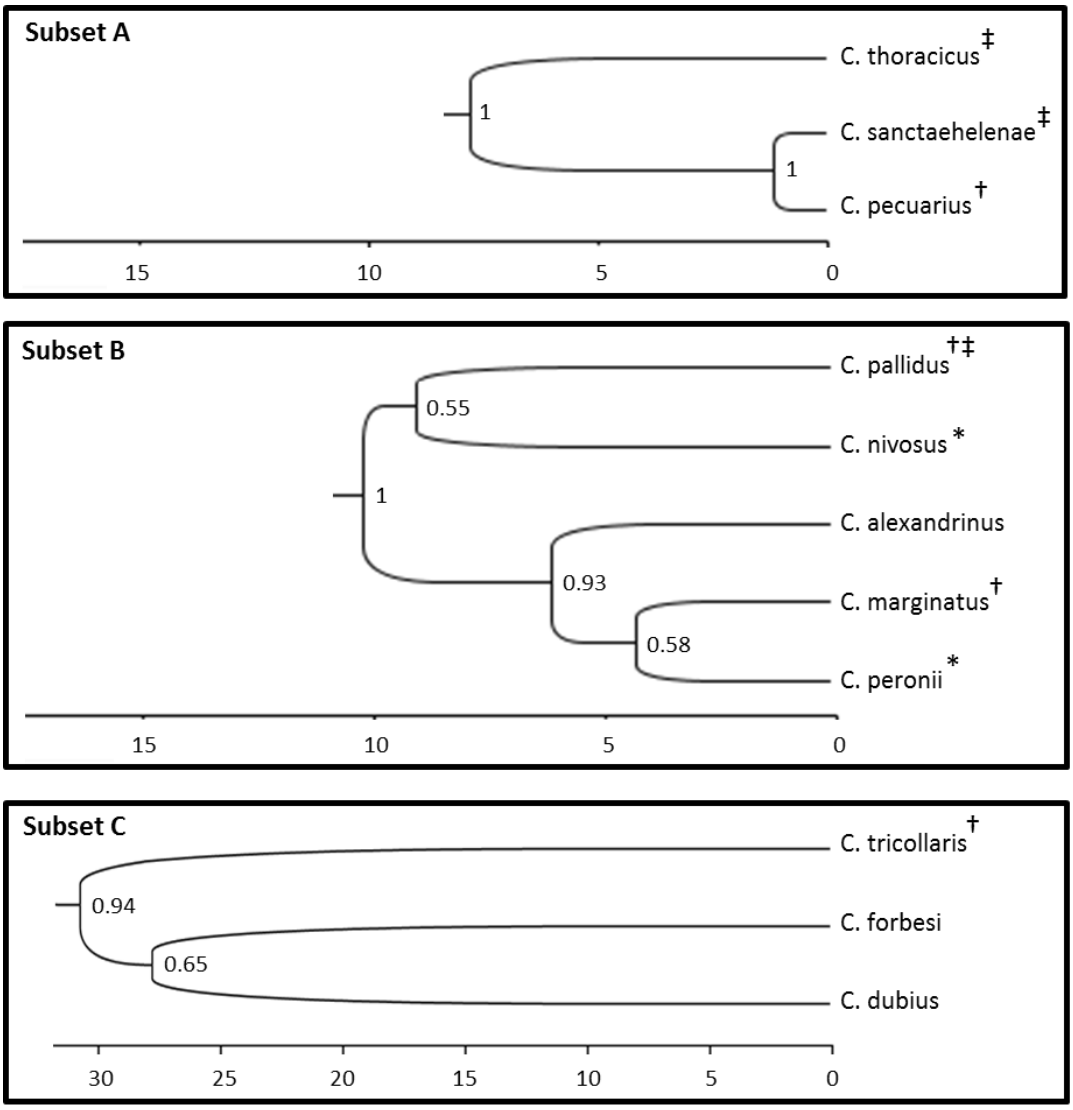

Figure 1: The *Charadrius* plovers of Africa, divided into three genetically distinct species subsets based on previous global analyses (Chapter 5). Nodes are labelled with posterior probabilities and scale axes indicate estimated molecular clock divergence times (million years ago; Mya) based on global analyses. * = non-African species; † = multiple populations sampled; ‡ = species of conservation interest. *C. ruficapillus* did not emerge within Subset B in our previous global analyses, but is included here in this study following earlier classification (Hayman et al. 1986; Sibley & Monroe 1990).

C. peronii, toe-pad samples were collected from museum specimens at the Natural History Muesum, Tring.

DNA extraction, amplification and sequencing

Full details of DNA extraction, PCR amplification, sequencing and alignment methods are included in Chapter 5.

Six genes were targeted for Polymerase Chain Reaction (PCR) amplification and sequencing including two mitochondrial loci: *COI* (cytochrome oxidase I) and *ND3* (NADH dehydrogenase subunit 3), and four nuclear loci: *ADH5* (alcohol dehydrogenase 5), *FIB7* (*β-fibrinogen intron 7), Myo-2/3* (Myosin-2/3) and *RAG1* (recombination activating gene 1). These genes were selected based on their previous utility in species-level avian phylogenies (Johnson *et al.* 2009; Chesser 1999; Fain *et al.* 2007; Fain & Houde 2007; Erikson *et al.* 2003). For DNA extracted from blood samples, 'universal' avian primers were used whereas, since degradation of DNA was expected from museum toe-pad samples, we utilised primers targeting shorter regions of each gene (details in Chapter 5). Cycle sequencing was performed by GenePool Laboratory (NBAF-Edinburgh).

Phylogenetic analyses

Best-fit nucleotide substitution models were selected based on Akaike's Information Criterion (AIC) in MrModelTest 2.3 (Nylander 2004). Phylogenetic analyses were performed in *BEAST 1.7.5 (Heled & Drummond 2010) using the NBAF-Sheffield computing cluster and also XSEDE on the CIPRES (Cyberinfrastructure for Phylogenetic Research) gateway (Miller *et al.* 2010; for full details see Chapter 5) for each of the three species Subsets independently (Subsets A – C, Fig. 1). To enable estimation of divergence times, the substitution rate for ND3 was set to 0.00233 based on Pereira and Baker (2006). For the remaining genes, ucld means (mean rates under the uncorrelated log-normal relaxed clock) were estimated with uniform distribution, range 0 - 0.1 and initial values of 0.001 or for COI, 0.00799 based on Pereira and Baker (2006). The Markov chains were run for 300 million generations with sampling every 15,000 generations.

*BEAST output was assessed for convergence to stationary distributions by inspection of the MCMC traces of samples in Tracer v1.5 (included in BEAST package). Summary of the posterior distribution of *BEAST trees and identification of the maximum clade credibility (MCC) tree for each species set was conducted using TreeAnnotator 1.7.5 (Drummond & Rambaut 2007) with burn-in values of 10% and

median node heights. *BEAST models were analysed four times to determine if the independent runs converged on the same posterior distribution. The MCC tree was visualised in FigTree v1.4.0 (available at http://tree.bio.ed.ac. uk/ software/figtree/).

Results

Sequence characteristics

The full sequence length of all six concatenated genes was 4,295 base pairs. For DNA extracted from museum toepad samples, the length of sequence data amplified was 1,343 base pairs. For each of the four nuclear and two mitochondrial loci, sequence length and nucleotide substitution models are listed in Chapter 5 (Table 4). Levels of nucleotide diversity within each Subset are presented in Table 2, namely: π (mean number of nucleotide differences per site; Tajima 1983), s (number of segregating sites), Θ_w (population mutation rate parameter; Watterson 1975; analysed in DNAsp v5, Librado & Rozas 2009) and mean percentage pairwise identities for each gene. Additiontally, percentages of sequence divergence for *COI* (the DNA 'barcode' gene) are presented (estimated in Geneious 6.1.6) in Table 3, representing population-level estimates of phylogenetic divergence (four species) as well as divergence levels with each Subset.

Species-level relationships

Full details of divergence times with confidence intervals (95% highest posterior density, HPD) for each node, based on molecular clock estimates, are included in Table 4 (node labels in Fig. 3).

Within **subset A** (Fig. 2a), the Kittlitz's plover *C. pecuarius* was supported as the closest relative of the 'critically endangered' St Helena plover *C. sanctaehelenae*, with molecular clock estimation placing divergence at approximately 1.4 Mya (95% HPD 0.2 – 4.8). The Madagascar plover *C. thoracicus* diverged approximately 5M years earlier, with the most recent common ancestor (MRCA) of the group living approximately 6.6 Mya (95% HPD 0.5 – 26.6). Overall, this subset exhibited low levels of nucleotide diversity relative to the other subsets studied (π = 0.00517, Table 2; 3.7% *COI* sequence divergence)

For **subset B** (Fig. 2b), the MRCA of the group was estimated at 9.7 Mya (95% HPD 3 – 20), with the ancestral red-capped plover diverging from the other species at this time. The other five species became genetically differentiated much more recently, approximately 4 Mya (95% HPD 1.5 – 8).

The genetic differentiation between the three species in **subset C** (Fig. 2c) was greater than that of the other two subsets, with divergence times of approx. 10 Mya (95% HPD 2.8 – 20.7) for *C. forbesi* and *C. tricollaris* and approx. 12 Mya (95% HPD 2.1 – 16.7) for divergence of this lineage from the ancestor of *C. dubius*.

Population-level relationships

White-fronted plover: The Madagascar, Namibia and South Africa populations of white-fronted plovers, *C. marginatus*, exhibited relatively high levels of genetic differentiation (3.5% *COI* divergence). Molecular clock data suggested the Malagasy population split from the included mainland populations approx. 1.8 Mya (95% HPD 0.6 – 3.9; COI).

Chestnut-banded plover: There also appears to be strong genetic differentiation among the chestnut-banded plovers (*C. pallidus)* of Namibia, and those of Kenya and Tanzania (3.7% *COI* divergence). Individuals of Kenya and Tanzania exhibited very little genetic differentiation (1.8% *COI* divergence) and formed a single coherent genetic cluster, distinct from the chestnut-banded plovers of Namibia, with an estimated divergence time of approximately 1 Mya (95% HPD 0.2 – 2.7).

Three-banded plover: The three-banded plovers, *C. tricollaris*, of Madagascar and Kenya exhibited genetic differentiation consistent with genetic isolation and colonisation of Madagascar nearly 1 Mya (Table 4; Fig. 3; 3.4% *COI* divergence).

Kittlitz's plover: In comparison with the clear genetic divergence of *C. marginatus*, *C. pallidus* and *C. tricollaris* populations, populations of Kittlitz's plover *C. pecuarius* exhibited lower genetic differentiation (1.6% *COI* divergence; dated at 1 Mya, 95% HPD 0.2 – 3.6). Additionally, phylogenetic relationships between populations did not match geographical location: the Kenyan population grouping with Namibian, South African with Senegalese.

Table 1: Sampling locations and breeding locations of included species, ordered by species set. Sample size per population = 3 individuals. † = samples collected from museum specimens. * = non-African species.

Species	Common name	Sampling locations	Breeding location	Subset
C. pecuarius	Kittlitz's plover	Kenya, Namibia, Madagascar Senegal, South Africa,	Africa	A
C. sanctaehelenae	St Helena plover	St Helena	Southern Africa	A
C. thoracicus	Madagascar plover	Madagascar	Southern Africa	A
C. alexandrinus	Kentish plover	Cape Verde	Eurasia/ North Africa	B
C. marginatus	White-fronted plover	Namibia, Madagascar, South Africa	Southern Africa	B
*C. nivosus**	Snowy plover	Mexico	North/ South America	B
C. pallidus	Chestnut-banded plover	Kenya, Namibia, Tanzania	Southern Africa	B
C. peronii †	Malaysian plover	Borneo	South-East Asia	B
*C. ruficapillus**	Red-capped plover	Australia	Australasia	B
C. dubius	Little Ringed plover	China	Eurasia	C
C. forbesi †	Forbes' plover	Nigeria	Central Africa	C
C. tricollaris	Three banded plover	Kenya, Madagascar	Southern Africa	C

Table 2: Properties of sequence data for Subsets A, B and C including mean nucleotide diversity (π), number of segregating sites (s) and the mutation rate parameter (Θ_W) as well as levels of sequence similarity (mean % pairwise identity) for each gene.

Subset	π	s	Θ_W	Mean % pairwise identity					
				COI	ND3	ADH5	FIB7	Myo2	RAG1
A	0.0052	87	0.00495	99.1	99.0	99.2	98.7	99.3	99.9
B	0.0142	214	0.01156	91.9	95.7	98.8	98.6	98.5	99.7
C	0.0193	182	0.01272	94.1	96.4	89.1	98.6	99.1	99.7

Table 3: Levels of *COI* sequence divergence for four plover species with data from multiple populations, and among all species within each subset.

Subset	Species	*COI* % sequence divergence
A	*C. pecuarius*	1.6
	C. pecuarius/ C. sanctaehelenae	1.9
	All species	3.7
B	*C. marginatus*	3.5
	C. pallidus	3.7
	All species	15.8
C	*C. tricollaris*	3.4
	All species	11.2

Table 4: Divergence time estimates (95% HPD) for each node based on mitochondrial molecular clock data from Pereira & Baker (2006). See Fig. 3 for node labels.
* = nodes with 95% HPD range overlapping the present day (0 Mya).

Subset	Node	95% HPD
A	1	0.5 – 26.6
	2	0.2 – 4.8
	3	0.2 – 3.6
	4*	0.0 – 2.2
	5	0.1 – 1.9
	6*	0.0 – 1.1
B	7	3.0 – 20.0
	8	1.5 – 8.1
	9	1.1 – 6.8
	10	0.1 – 2.6
	11*	0.0 – 0.2
	12	0.9 – 5.7
	13	0.6 – 4.0
C	14	2.8 – 20.7
	15	2.1 – 16.8
	16	0.1 – 1.6

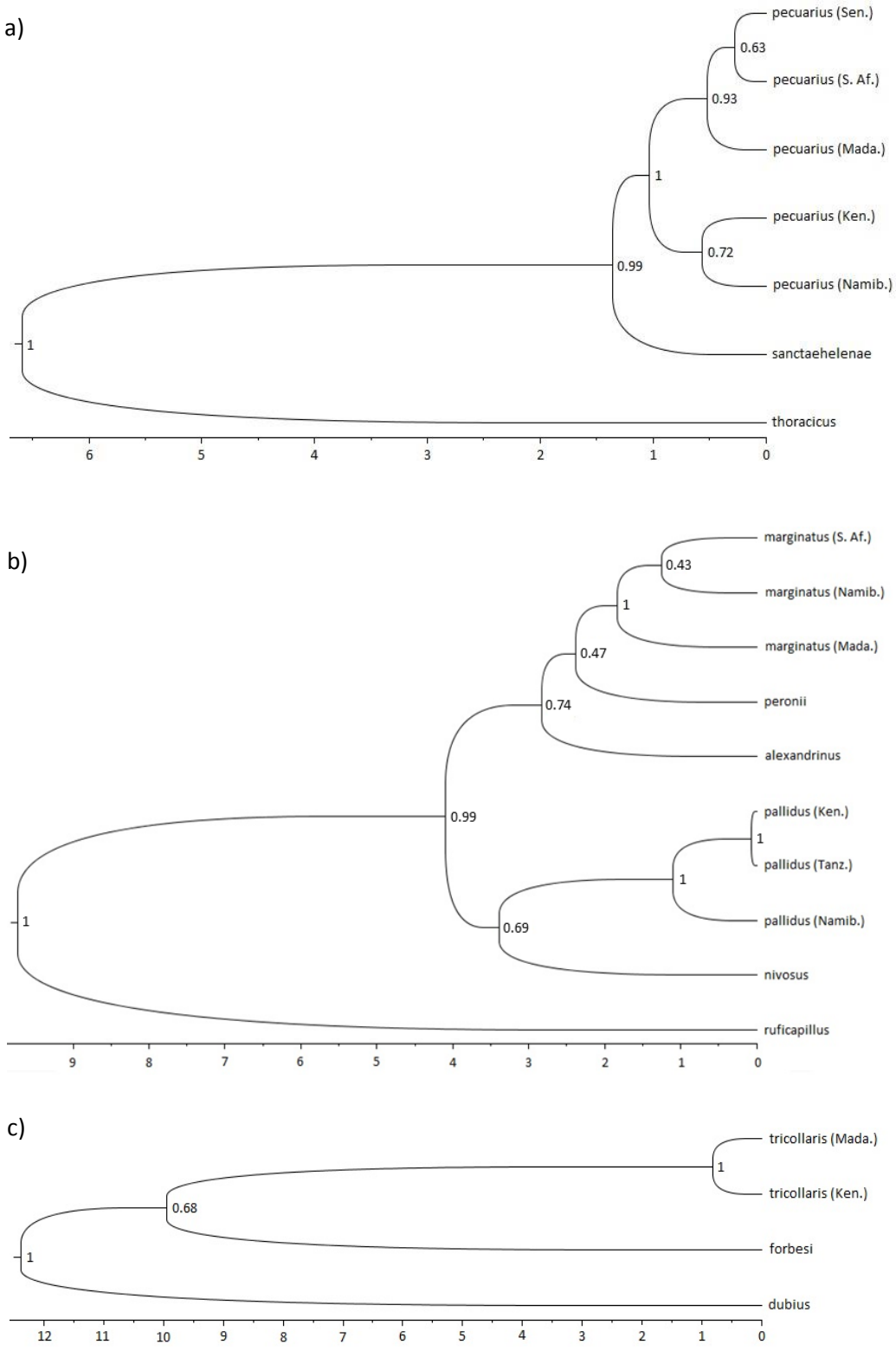

Figure 2: Maximum clade credibility trees for the three African species subsets: a) Subset A, b) Subset B, c) Subset C. Details of species in each subset are given in Table 1. Nodes are labelled with posterior probabilities. Scale axes represent estimated molecular clock divergence times in million years ago (Mya).

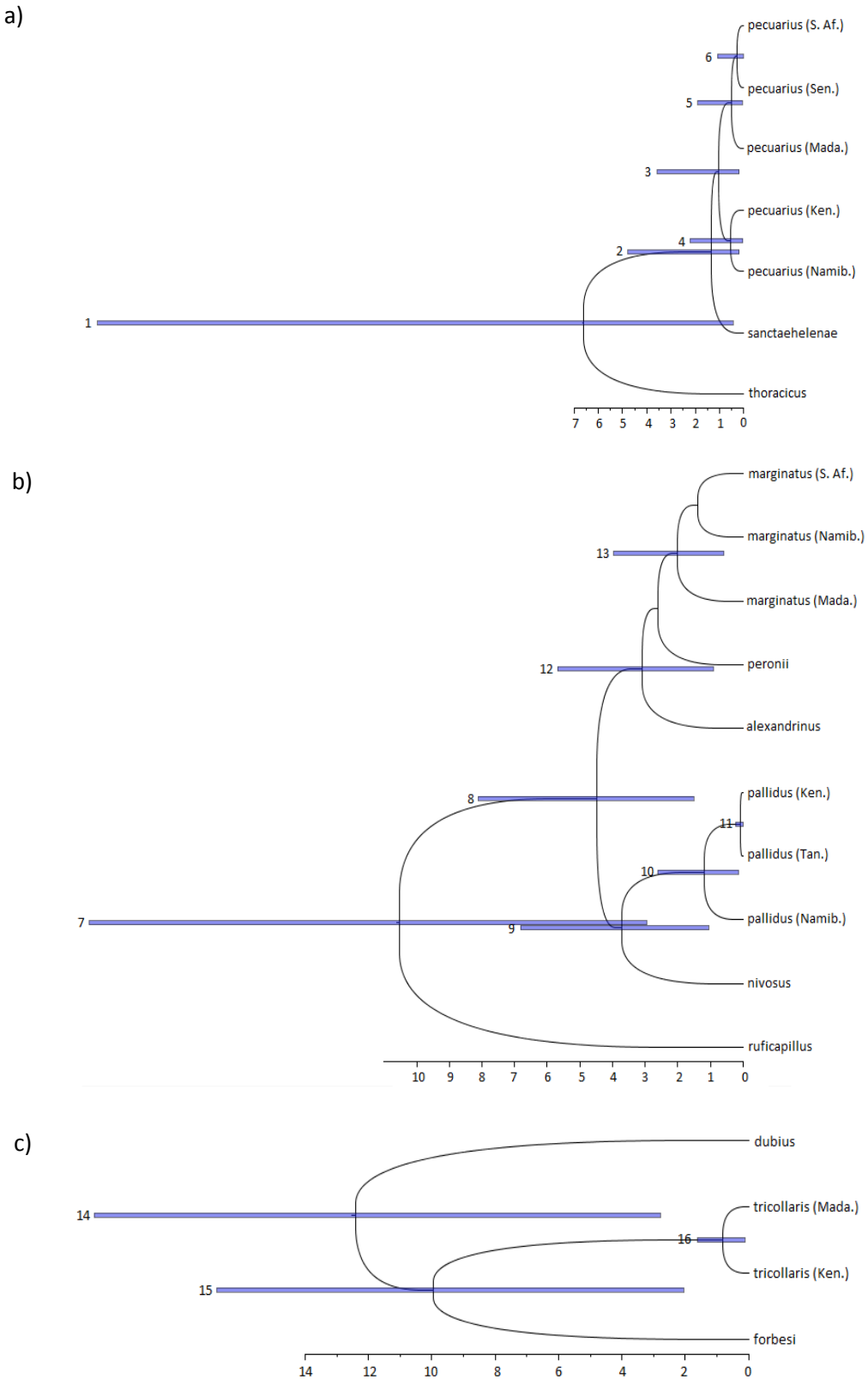

Figure 3: Maximum clade credibility trees for the three African species subsets: a) Subset A, b) Subset B, c) Subset C. Details of species in each subset are given in Table 1. Node bars represent confidence intervals on divergence times for each node with posterior probability >0.5. Scale axes represent estimated molecular clock divergence times in million years ago (Mya).

Discussion

Species-level evolutionary history

Our second assessment of species-level phylogenetic relationships within the three lineages of *Charadrius* plovers that currently breed in Africa matched those outlined in our global phylogeny (Fig. 1) in all but one case. Namely, the chestnut-banded plover *C. pallidus,* was here identified as sharing more recent common ancestry with the snowy plover *C. nivosus* than the Kentish plover *C. alexandrinus* rather than vice versa. Based on their current geographic locations, it is unlikely that the African *C. pallidus* emerged from the American species, therefore we suggest that ancestral populations of C. nivosus may once have been more widespread, but have now become restricted to America, and, in Africa been replaced entirely be C. alexandrinus and *C. pallidus*. In order to fully resolve the phylogenetic relationships within this group, further research, including a greater number of genetic markers, will be necessary.

Africa was identified as the location of most recent speciation within the genus *Charadrius* (Chapter 5). In particular, colonisation of islands seems to have been an important step in the development of genetic isolation, notably on the islands of St Helena and Madagascar. Our results suggest the MRCA of the 'critically endangered' St Helena plover *C. sanctaehelenae* and widespread Kittlitz's plover, *C. pecuarius,* colonised St Helena just 1 Mya. Based on this estimated divergence time, these two species are the most recently differentiated sister species within the genus. The St Helena plover warrants its species status due to the phenotypic differences identified with the Kittlitz's plover – the St Helena plover is a third larger in size and exhibits reduced tan colouration on the breast (del Hoyo *et al.* 1996; Rowlands *et al.* 1998).

The Madagascar plover, *C. thoracicus*, appears more phenotypically similar to the Kittlitz's plover than does the St Helena plover, and was therefore placed as the closest relative of the Kittlitz's plover in Livezey's (2010) phenotypic phylogeny. However, our results suggest that the Madagascar plover emerged earlier in the history of the group, approximately 6.6 Mya. Both the Kittlitz's plover and Madagascar plover inhabit Madagascar today and we suggest that the ancestral Madagascar plover was the first to colonise Madagascar with the modern Kittlitz's plover following much later (<1 Mya). These results are in line with patterns observed among other taxa, including the bat genus *Triaenops* (Russell *et al.* 2008) and the small minnow mayflies (*Ephemeroptera: Baetidae*; Monaghan *et al.* 2005), with multiple dispersal events taking place from

mainland Africa towards Madagascar, resulting in the emergence of several independent lineages here during the evolutionary history of the genus.

Until recently, the Kentish plover *C. alexandrinus* (of Eurasia) and snowy plover *C. nivosus* (of N. and S. America) were considered conspecific (Küpper *et al.* 2009). We here support their re-classification as distinct species, and estimate divergence at approximately 4.1 Mya. Furthermore, some authors placed these species within a superspecies including the white-fronted plover and red-capped plover (Hayman *et al.* 1986, Sibley and Monroe 1990). Our previous global phylogeny questioned the placement of the red-capped plover, with discordance between gene trees leading to low support for a placement outside the species set (Chapter 5). In this study, the red-capped plover appeared as the first species to become differentiated from the group, separating from its sister species approximately 10 Mya - more recently than our previous estimate of 23 Mya (Chapter 5). Our results suggest the additional inclusion of the chestnut-banded plover (*C. pallidus*) and the Malaysian plover (*C. peronii*) within this superspecies (see also Rheindt *et al.* 2011). The phenotypic phylogeny of Livezey (2010) also supported the inclusion of *C. peronii* within the superspecies, but in contrast to our results, placed *C. pallidus*, *C. marginatus*, and *C. ruficapillus* in a separate lineage, highlighting the mismatch between phenotype and genotype among the *Charadrius* plovers.

The three species of subset C, *C. forbesi*, *C. tricollaris* and *C. dubius* appear more distantly related than species within the other two subsets with divergence approximately 10 Mya (*C. tricollaris* and *C. forbesi*) or earlier (*C. dubius*). It is therefore surprising that *C. forbesi* and *C. tricollaris* occupy almost entirely non-overlapping ranges in the north of Africa in the present day. Within the global phylogeny (Chapter 5), these species were placed within the largest and oldest clade that began to diversify approximately 26 Mya with lineages resulting in the modern ringed plovers. It is likely that their common ancestor was the first *Charadrius* plover to colonise Africa.

Overall, we observe several differences between phenotypic (Livezey 2010) and genotypic levels of similarity among the African plovers. Phenotypically, *C. forbesi* and *C. tricollaris* were placed as separate from the genus *Charadrius,* whereas our molecular data place them well within the genus (Chapter 5). Recently emerging species, according to molecular phylogenetic data, exhibit great phenotypic differences with their closest sister species (e.g. St Helena and Kittlitz's plover). In contrast, species that exhibit strong similarity in phenotypic traits may have been genetically differentiated for a considerably longer period of evolutionary time (e.g. three-banded plover and Forbes's

plover). Phylogenetic data are more informative in determining the evolutionary history of species than phenotypic data. Rates of genetic change are generally more constant than rates of phenotypic change (Hillis 1987), and based on phenotypic data alone, it can be more difficult to distinguish homoplasy (by convergent or parallel evolution) from evolutionary homology (Wake *et al.* 2011; Nixon & Carpenter 2012).

Although relatively high levels of support (>0.9) were recovered for the majority of nodes, posterior probabilities were below 0.9 for 7 of the 18 nodes analysed across the three maximum clade credibility trees constructed for the African plover species subsets. It is possible that a larger quantity of sequence data may have enabled the recovery of more well-supported trees. However, the improvement in terms of resolving trees with greater levels of support may be small relative to the significant costs involved in obtaining additional sequence data. Studies on the quantity of sequence data required to resolve molecular phylogenies have provided no clear guidance, but have highlighted the fact that this will depend greatly upon factors such as the number of taxa, the level of divergence among taxa, the analysis method and the level of support required, with between 2,000 and 6,000 bp providing sufficient information in the majority of cases (Moret *et al.* 2002; Spinks *et al.* 2009). Furthermore, since multigene datasets, such as those included in this study, often contain conflicting phylogenetic signals, analyses may recover nodes with poor support even with the inclusion of extensive sequence data (Spinks *et al*. 2009). The quantity of sequence data analysed in this study was above minimum recommendations (2,000 bp, Moret et al. 2002) therefore we suggest that an increase in the quantity of sequence data included in analyses would not have been likely to greatly improve the quality of our results.

Population-level biogeography

We have already highlighted Madagascar as an important location in the formation of plover species (see above). At the population level, the importance of Madagascar again emerged, with genetic differentiation between mainland and Malagasy populations of the white-fronted plover *C. marginatus* and three-banded plover *C. tricollaris*. Madagascar is located in the Indian Ocean, 250 miles from mainland Africa. This geographic distance is associated with genetic distance for a variety of species, as populations of sedentary species become isolated from their conspecifics on the mainland (Monaghan *et al.* 2005; Russell *et al.* 2008) and the sea provides an effective barrier for gene flow (Küpper *et al.* 2012). This reduction in gene flow between

populations, over evolutionary time, can facilitate allopatric speciation and the emergence of new species.

The chestnut-banded plovers, *C. pallidus*, are currently classified into two subspecies, *C. p. pallidus* of southern Africa and *C. p. venustus* of Kenya and Tanzania. Our results support this classification, with individuals of Kenya and Tanzania exhibiting little genetic differentiation, representing a single population and subspecies distinct from the chestnut-banded plovers of Namibia, with an estimated divergence time of approximately 1 Mya. These findings suggest that the two subspecies are not connected by concurrent gene flow. The subspecies *C. p. venustus* is of particular conservation interest consisting of 6,338 individuals, with 84% of the population occupying just three sites: Lakes Manyara and Natron, Tanzania and Lake Magadi, Kenya (Simmons *et al.* 2007). The nominate subspecies is also of conservation concern, though larger in size at 11,486 birds, 96% of the population were counted at just two sites: Walvis Bay and Sandwich Harbour, Namibia (Simmons *et al.* 2007).

Among the populations of white-fronted plover, *C. marginatus*, included in analyses, two (Namibia and South Africa) are currently classified into one subspecies, *arenaceus,* whilst the third is classified as a separate subspecies on Madagascar, *C. m. tenellus*. In support of this, the Malagasy population shows differentiation consistent with divergence from the included mainland populations approx. 2 Mya. Additionally, based on the level of genetic differentiation observed between Namibian and South African populations of *arenaceus,* subspecies status may be appropriate. Further investigation, including data from populations across Africa would be necessary to elucidate this. Evidence of genetic differentiation between Madagascar and mainland Africa was also found within the three-banded plover, again supporting the classification of two subspecies *C. tricollaris tricollaris* on mainland Africa, *C. tricollaris bifrontatu*s on Madagascar (Delany *et al.* 2009).

Of the three widespread species for which multiple populations were included, the Kittlitz's plover displays lower genetic differentiation between populations than the chestnut-banded plover, white-fronted plover or three-banded plover. These results concur with previous taxonomy for the Kittlitz's plover, identifying just one subspecies (*C. p. pecuarius*; Delany *et al.* 2009) across Madagascar and much of mainland Africa, with the exception of one resident population in northern Egypt, (*C. p. allenbyi*). Furthermore, phylogenetic relationships between populations did not match geographical location (Kenyan populations with Namibian, South African with Senegalese), suggesting that dispersal occurs between breeding populations over a large scale. Further research,

including more populations of Kittlitz's plovers will be necessary to determine the mainland origin of the modern Malagasy population of this species.

Though there may be many factors involved in maintaining connectivity between populations and driving dispersal over a large scale (e.g. local population densities, resource availability, environmental stochasticity or avoidance of inbreeding), in the case of the Kittlitz's plover, one possible factor that distinguishes this species from the other species studied is their mating system (Küpper *et al.* 2012). The Kittlitz's plover exhibits a polygamous mating system whereas the other three species are monogamous. Monogamous species often exhibit higher levels of philopatry and are more site-faithful than polygamous species since polygamous individualsmay travel large distances to produce broods in multiple locations within and between breeding seasons (Reynolds & Cooke 1988). Such dispersal acts to increase gene flow between polygamous populations, in contrast to monogamous species that tend to be more site-faithful, often returning year after year to the same breeding site. We suggest that this link between mating systems and genetic diversity warrants further investigation across multiple populations of species with differing mating systems.

Another area for further investigation concerns the closely-related 'critically endangered' St Helena plover and the Kittlitz's plover. Research into the similarities and differences between the two species may reveal whether particular intrinsic biological traits (e.g. migratory status, breeding system or habitat specialisation) have placed the endangered species at risk, or whether the present conservation status of the St Helena plover is related to extrinsic conditions only (as outlined in McCulloch 2009; Burns *et al.* 2013).

In the current study, we analysed mitochondrial and nuclear DNA together in order to gain an overall view of the evolutionary history of, and phylogenetic relationships among, the *Charadrius* plovers of Africa. For future investigations, such as those proposed above, more in-depth analyses of maternally-inherited mitochondrial DNA data have the potential to identify the existence of sex-biased dispersal patterns. Such information may be central to understanding the evolution of species with differing mating systems, as well as investigating the factors underlying the evolutionary differences between widespread and endangered species.

Conclusion

Our results provide high levels of support for the species-level relationships presented in our global phylogeny (Fig. 1; Chapter 5). At the population level, we identified genetic

differentiation supporting the classification of multiple subspecies for *C. pallidus*, *C. marginatus* and *C. tricollaris*, but lower genetic differentiation among populations of *C. pecuarius*. Both species- and population-level analyses highlight Madagascar as an important location in genetic diversification among the *Charadrius* plovers. We suggest that levels of genetic differentiation between populations may be linked to behavioural traits such as mating systems and dispersal between breeding sites. We encourage further studies addressing *how* genetic differentiation has developed between populations of *Charadrius* plovers. Additionally, focusing on the 'critically endangered' St Helena plover, we propose modelling the demographic history of the species in relation to mainland populations of its closest sister species, the Kittlitz's plover. By conducting analyses using approximate Bayesian computation (ABC) and principal component analysis (PCA), and by including genetic data from ancient museum specimens, as well as data from historic population censuses on St Helena, this work has the potential to reveal new insights on the evolutionary history, long-term population size and genetic diversity of this endangered island species.

Acknowledgements

For acquisition of samples, we thank: Bernard Amakobe, Neil Baker, Marcela Beltrán, Fiona Burns, Balazs Kaholek Sylvester Karimi, A. Morrison, Christine Ochieng, Jorge Parra, H. King Robinson, W. Serle, Bela Tokody, Pavel Tomkovich, Mike Weston and Sama Zefania. We also thank Mark Adams, Robert Prys-Jones and Douglas Russell for sample collection from museum specimens at the Natural History Museum, Tring. Sequence analysis was supported by NERC-Biomolecular Analysis Facility at the University of Edinburgh and at the University of Sheffield. This study was funded by the NERC as part of the PhD research of NdR. CK was supported by a Marie Curie IEF postdoctoral fellowship. Permits for fieldwork and collection of samples were provided by the national agencies in each country. Lastly, thanks to Michael Hutchinson for editorial assistance.

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7 Discussion

Breeding system evolution

Understanding the mechanisms driving the evolution of breeding systems is one of the central goals in behavioural and evolutionary ecology. Empirical research is essential to determining the importance of proximate and ultimate mechanisms in wild populations (Emlen & Oring 1977; McGraw *et al.* 2010). Firstly, studying microevolutionary variation in mating systems and parental care among closely-related populations with flexible breeding systems, such as the *Charadrius* plovers, could enable us to elucidate the relative importance of these factors without confounding phylogenetic effects (Brashares *et al.* 2000; Schradin *et al.* 2012; Schradin 2013). Secondly, investigating breeding system variation across higher-level taxa, within a more integrative phylogenetic framework, has the potential to reveal new insights on the macroevolutionary processes involved in breeding system evolution across taxa. The work presented within this thesis has driven forward research in both the first and second lines of study.

In Part I, I investigated the role of sex biases among offspring in breeding system evolution on a microevolutionary scale. The main findings were as follows:

- Using molecular sex determination (Chapter 2), I identified higher survival levels among sons than daughters under particular conditions (broods cared for by single parents) in one population with largely male care, whereas in one population where males and females care for offspring equally, females had higher levels of survival than males (Chapter 3).
- I found that male plover chicks were either larger than females at hatching, or exhibited faster growth than females in two populations with predominantly male parental care, whereas no sex differences in hatching size or growth were present in two populations with more balanced parental care roles (Chapter 4).

These results provided some support for theoretical hypotheses linking sex biases among offspring to sex-biased parental care roles. Where present, sex differences in growth and survival were in the predicted direction: sons grew faster and survived better than daughters in populations with male-biased adult sex ratios, female-biased mating opportunities and male parental care, but not in populations with equal male and female parental care. However, sex differences were not consistent across populations, and varied within and between years, suggesting that interactions between social and physical environmental conditions were important in the manifestation of sex biases among offspring, and the establishment of sex ratio biases in populations with largely male parental care.

The importance of ecological and environmental factors in the evolution of social behaviours has been extensively studied (Wilson 1975; Krebs & Davies 1993; Lacey & Wieczorek 2003; Schradin *et al.* 2012; Solomon & Keane 2012). For example, extreme temperatures (e.g. Kosztolányi *et al.* 2011), predation risk (e.g. Townshend *et al.* 1985) and variation in the spatial and temporal distribution of resources (e.g. Hourigan 1989) have been suggested to influence parental care and the evolution of social systems across taxa. However, research suggests that ecological factors alone are usually not sufficient to explain the evolution of parental care (Klug & Bonsall, 2010; Klug *et al.* 2012). My work suggests that social environment is also important in driving breeding system variation, and that integration of empirical research in these areas will be necessary in order to form a more comprehensive understanding of microevolutionary variation in breeding systems.

Furthermore, my research is part of a growing body of evidence for the importance of offspring sex ratios in the evolution of social behaviour (McNamara *et al.* 2000, Hardy 2002, Kokko & Jennions 2008; West 2009; Jennions & Kokko 2010; Liker *et al.* 2013). However, studying the development of sex ratio biases among offspring provides only an incomplete picture. Offspring sex ratios are now relatively easy to quantify in wild populations due to the availability of molecular sex determination techniques (Chapter 2). For a more complete picture, it is important, firstly, to quantify variation in sex ratios throughout the life history of individuals. Generating reliable estimates of adult sex ratio (ASR) in wild populations is not easy as there are often differences in the sighting or trapping of males and females (Domenech & Senar, 1998; Newson *et al.* 2005; Amrhein *et al.* 2012). Recently, methods for demographic modelling, such as perturbation analyses (Veran & Beissinger 2009; Kosztolányi *et al.* 2011), have been developed to explore ASRs as well as operational sex ratios (OSRs; among sexually

active individuals). Secondly, researchers must aim to quantify both social and genetic mating systems as, for example, though 90% of birds are socially monogamous (Lack 1968), many of these species are not genetically monogamous (Griffith & Montgomerie 2002; Griffith *et al.* 2003; Westneat & Stewart 2003). Thirdly, researchers must aim to empirically address the causative link between sex ratios and breeding systems, beyond mere correlative results. Experimental approaches have the potential to provide insights here. For example, in populations with male-biased OSRs, mating opportunities should be higher for females than males, and hence, where parents are not constrained to biparental care (e.g. the care needs of offspring can be attended to by a single parent), females would be expected to desert more often than males, leaving their partner 'holding the babies' (Székely *et al*. 1999; McNamara *et al*. 2000; Jennions and Kokko 2010). One approach is to conduct experimental manipulations in order to quantify mating opportunities for single males and females in monogamous and polygamous populations (Appendix 1).

Combining these three approaches in future research provides the opportunity to more rigorously test theoretical predictions on the link between sex ratios and breeding system evolution. Additionally, with a view to further elucidating the mechanisms involved, researchers should aim to integrate this work with data on the genetic, neurological and hormonal bases of social behaviour in wild populations (Moore *et al.* 2010).

Evolutionary history and phylogeography

Unravelling the evolutionary history and phylogeography of species is of fundamental importance in broader-scale macroevolutionary studies on the evolution of behavioural, morphological and life history traits. The phylogenetic approach is now widely utilised in social behaviour research (Kamilar & Cooper 2013; Kappeler *et al.* 2013; Thierry 2013). However, without a global molecular phylogeny of the genus *Charadrius*, evolutionary studies on these diverse shorebirds have been limited in scope.

In Part II of this thesis, I explored the global phylogeographic history and evolutionary origins of the *Charadrius* plovers, providing a phylogenetic framework that will be central to future studies on the genus. The main research findings were as follows:

- I presented the first global, species-level molecular phylogeny of the genus *Charadrius* and outlined six discrete evolutionary lineages (*Clades I – VI*; Chapter 5).

- I identified the Northern hemisphere (North America, Europe and Siberia) as the origin of the ancestral *Charadrius* species between 19.2 and 36.6 million years ago (Chapter 5).

- I suggested that early evolutionary diversification occurred due to shifts in the range of ancestral Northern hemisphere species in response to global cooling during the Miocene period, driving populations southwards, towards lower latitudes, and into the Southern hemisphere (Chapter 5).

- I identified the widespread Kittlitz's plover (*C. pecuarius*) as the closest sister species to the 'critically endangered' St Helena plover (*C. sanctaehelenae*; 0.2 – 2.4 Mya divergence) and also the 'vulnerable' Madagascar plover (*C. thoracicus;* 2.8 – 10 Mya divergence; Chapter 6).

- I identified genetic structure consistent with subspecies status among African populations of the white-fronted plover (*C. marginatus*), three-banded plover (*C. tricollaris*) and chestnut-banded plover (*C. pallidus*), but not the Kittlitz's plover (Chapter 6).

The proposed importance of Miocene global cooling on early diversification of the genus *Charadrius* out of the Northern hemisphere (North America, Europe and Siberia) represents a novel theory on the evolutionary origins of the genus. This work contributes to the broader field of historical biogeography, contributing to knowledge on global patterns, range shifts and the diversification of Northern hemisphere species in response to paleoclimatic events. The phylogeographic origin of the *Charadrius* plovers is similar to that hypothesised for large mammalian carnivores (Hunt 2004) and other animal taxa (Zachos *et al.* 2001; Maguire & Stigall 2008; Schweizer *et al.* 2011).

Ice ages have traditionally been proposed as the cause of divergence between sister taxa (Avise & Walker 1998) and the role of Tertiary and Pleistocene glaciation events in the diversification of Holarctic shorebird species was outlined over 50 years ago, in a classical study by Larson (1957). This study examined the systematics of recent and fossil forms and highlighted particular glacial refugia that may have been important in the geographical distribution and evolution of different climate-adapted 'forms' of *Charadrii* (temperate, warm and cold-adapted species). More recently, this topic was revisited by Kraaijeveld & Nieboer (2000) who focussed on the influence of recent Pleistocene glaciation (within the last 250,000 years) in the distribution of modern shorebird populations and suggested that populations of low arctic species became

genetically differentiated, divided by ice sheets, during glacial periods, leading to the formation of subspecies of species such as the dunlin (*Calidris alpina*). My work suggests that these processes were important, not only in recent population-level differentiation among shorebirds, but in the initial diversification of the genus *Charadrius* during the Miocene period and the colonisation of the southern hemisphere.

Prior to the molecular genetic revolution (Clark & Pazdernik 2012), and throughout the 20th century, substantial research effort was devoted to identifying morphological characters (e.g. osteological or anatomical traits) that were informative in estimating the taxonomic relationships among shorebird species (Lowe 1922; 1933; Strauch 1978; Johnsguard 1981). These proposed relationships were much debated, for example, Strauch's (1978) classical study on 227 *Charadriiform* taxa focused on 70 osteological characters that were subsequently criticised and reanalysed several times, using modified data and updated methodologies, by Mickevich & Parenti (1980), Björkland (1994) and Chu (1995). Each reanalysis proposed alternative hypotheses on the major splits within the clade and monophyly of particular groups (e.g. *Scolopaci* and *Charadrii*; Björkland 1994).

Today, molecular genetic data dominate studies on the evolutionary history and phylogeography of extant taxa (Scotland *et al.* 2003; van Tuinen *et al.* 2004; Wiens 2004; Mayr 2011), though for many species without available molecular data, phenotypic data still provide the best estimate of phylogenetic history (e.g. molecular data not available for 3,330 of 9,993 species in first draft of 'complete' global bird phylogeny; Jetz *et al.* 2012). In recent years, phylogenetic data have been used to explore higher level phylogenetic relationships among the shorebirds (order *Charadriiformes*; approx. 350 species; Paton *et al.* 2002; Ericson 2003; Thomas *et al.* 2004; Baker *et al.* 2007; Fain & Houde 2007), however no global molecular phylogeny at the species level has been produced for the genus *Charadrius.* My research fills this gap, and concurs with the estimated age of the genus published by Paton *et al.* (2003; 17.8 – 38.6 Mya), placing the MRCA of the genus between 19.2 – 36.6 Mya (Chapter 5). Regarding evolutionary history, my work contrasts with the molecular phylogeny of Joseph *et al.* (1999) in suggesting a Northern hemisphere rather than Southern hemisphere origin. My results also differ from the recent phenotypic phylogeny of Livezey (2010) who placed species such as *C. tricollaris*, *C. forbesi* and *C. obscurus* within separate, non-*Charadrius* genera.

Lastly, my results revealed that the St Helena plover diverged from a common ancestor with the Kittlitz's plover approximately 1 million years ago. This evolutionary information is of value to conservationists aiming to identify the historical population

demography of the 'critically endangered' island species (IUCN 2013). Additionally, this phylogenetic data provides the opportunity to investigate similarities and differences in behavioural, physiological and life history traits between the 'critically endangered' and a closely related but more widespread species. For example, by utilising analytical approaches such as approximate Bayesian computation (ABC; Rubin 1984) and principal component analysis (PCA; Pearson 1901), studies have the potential to reveal: a) how the St Helena plover has adapted in relation to its island habitat; b) whether any intrinsic traits influence the vulnerability of the species to threats such as habitat loss and predation (McCulloch & Norris 2001; Burns *et al.* 2013) and c) whether such traits are unique to the St Helena plover or are also expressed in the Kittlitz's plover – or whether the present conservation status of the St Helena plover is largely related to environmental conditions (threats outlined in McCulloch 2009; Burns *et al.* 2013).

Breeding system evolution and phylogeography

Breeding systems and phylogeography should be tightly linked in the evolutionary history of populations, species and higher-level taxa. The interactions between breeding system and phylogeography among populations, on a microevolutionary scale, ultimately contribute to driving speciation and breeding system evolution on a macroevolutionary scale. Studying both microevolutionary and macroevolutionary processes therefore has the potential to reveal insights on the evolutionary history of phylogenetic groups, and the evolution of breeding systems within these groups.

How does phylogeography influence breeding systems?

Phylogeographic studies can reveal the historical and contemporary forces that have produced the current genealogical architecture and geographical distribution of populations and closely-related species (Avise 2009; Edwards *et al.* 2012; Chapter 1). Geographical distributions can influence the evolution of parental care and mating systems as well as other life history traits. For example, island populations with small population sizes and restricted ranges, such as the St Helena plover (*C. sanctaehelenae*) may be more likely to exhibit monogamy than their mainland relatives due to increased site-faithfulness in breeding areas and the small number of potential mates available. Covas (2012) examined reproductive patterns for island birds worldwide (306 species), taking into account the effects of latitude, and found that island colonisers exhibited largely bi-parental care (90% of island species in contrast to 81% of birds in general) and increased investment in young on islands. My research provides further support for

this relationship since two African island species, the St Helena plover and Madagascar plover (*C. thoracicus*), exhibit monogamy and biparental care in contrast to their closest sister species the Kittlitz's plover (Chapter 6), a species distributed widely across Africa that exhibits uniparental care and polygamy (by either the male or female). Additionally, in species with flexible breeding systems, biparental care is often exhibited in locations with extreme temperatures, where offspring require substantially different conditions for development than those of the ambient environment (Wilson 1975; Amat and Masero 2004; Kosztolányi *et al.* 2009; AlRashidi *et al.* 2011) and both parents may also be required in locations with high predation risk, limited availability of resources or intense competition (Brown *et al.* 2010).

The results of ancestral area analyses presented in this thesis (Chapter 5) suggest that modern plover species came to occupy Southern hemisphere continents secondarily, with ancestral species inhabiting Northern hemisphere regions. Once both social and genetic mating systems have been quantified across a wider range of species across the genus, researchers will have the opportunity to investigate how polygamy, monogamy and mixed mating systems have evolved within and between phylogenetic lineages in particular geographical regions (*Clades I – VI*; Chapter 5). Of particular interest is the question of whether mixed strategies and flexibility in breeding systems, such as that identified among populations of Kentish and snowy plovers (Lessels 1984; Warriner *et al.* 1986; Székely & Lessels 1993), are present across all lineages (indicating plasticity in social behaviours), or whether certain lineages have reached an evolutionarily stable state (indicating strong influence of genetic factors). Such research has implications for the evolutionary origins of mating systems and parental care among all birds, as the breeding systems and life history traits of plovers are very similar to those described for the very first ancestral bird species (Wesołowski *et al.* 2004; discussed in Chapter 1).

How do breeding systems influence phylogeography?

Mating systems can influence genetic diversity, genetic structure and gene flow between populations. Specifically, mating systems can influence genetic structure between populations as differences in levels of site-fidelity have been reported between monogamous and polygamous species (Greenwood 1980; Rohwer 1988). Polygamous individuals may disperse between breeding populations both within and between seasons whereas monogamous individuals are more philopatric (Rheindt 2011; Küpper

et al. 2012). Polygamy may therefore result in increased gene flow between breeding populations whereas monogamous species exhibit greater population-level genetic differentiation. In the case of polygamous species with high levels of dispersal, it is possible that genetic isolation, and ultimately speciation, are driven more by physical barriers whereas for monogamous species, the potential for genetic differentiation, and speciation, may exist between populations that are geographically closer together and are not necessarily restricted by barriers to dispersal.

My research on the *Charadrius* plovers, revealed greater genetic differentiation between populations of monogamous species such as the white-fronted plover (*C. marginatus*) and chestnut-banded plover (*C. pallidus*) than the polygamous Kittlitz's plover. Additionally, two monogamous African species with adjoining, but almost entirely non-overlapping ranges, the three-banded plover (*C. tricollaris*) and Forbes's plover (*C. forbesi*) seem to have remained geographically discrete after over 8 million years of divergence, suggesting very low levels of dispersal away from established sites.

To determine whether breeding systems are related to patterns of dispersal and genetic diversity, it will be necessary to conduct more in-depth investigations of genetic structure and population demography across many more populations of species with differing breeding systems. The phylogenetic tree produced in my study will enable the selection of appropriate *Charadrius* species to compare: for example, sister species with contrasting breeding systems, and pairs of such species replicated across the 6 major clades. Studies of genetic structure and population demography, using a large number of highly variable genetic markers (e.g. allozymes, mitochondrial or nuclear markers; see Vaseeharan *et al.* 2013) to construct high-density genetic maps (e.g. with ABC or PCA analyses) have recently provided new insights on spatiotemporal genetic structure among mammals (Warmuth *et al.* 2013), birds (Garroway *et al.* 2013), reptiles (Gassert *et al.* 2013), amphibians (Muir *et al.* 2013), deep sea fishes (Ritchie *et al.* 2013), plants (Emanuelli *et al.* 2013; Mandel *et al.* 2013) and fungi (De Backer *et al.* 2013). These approaches therefore have great potential in future studies on the influence of breeding systems on genetic diversity within a spatial context in the *Charadrius* plovers.

Future avenues of research

My PhD research has highlighted numerous interesting questions that warrant further investigation. In summary, three avenues of research appear particularly promising:

1. *Sex ratios and breeding systems, a holistic approach.* Researchers must aim to: a) quantify social and genetic mating systems; b) quantify firstly, *when,* and

secondly, *how,* sex ratio biases develop during the life history of individuals; c) elucidate the direct causative relationship between sex ratios and breeding system evolution - e.g. via experimental assessment of mating opportunities across multiple populations (Appendix 1).

2. *Genetic diversity and breeding systems.* Does greater genetic structure exist among populations of monogamous species than polygamous species, in line with predictions on higher levels of philopatry among monogamous than polygamous species?

3. *A socio-phylogeographic approach to breeding system evolution*. Using phylogenetic comparative analyses to study the relative influence and interactions between proximate mechanisms (genetic, hormonal, neurobiological, physiological) and ultimate factors (social and physical environment) in driving the evolution of breeding systems across taxa.

Overall, this thesis should be considered a stepping-stone, facilitating movement beyond microevolutionary studies on a small number of closely-related species, towards more integrative, macroevolutionary investigations, with a view to elucidating the mechanisms that drive breeding system evolution in wild populations. As well as addressing important questions on the evolution of breeding systems and the phylogeographic origins of the *Charadrius* plovers, this PhD has provided a much-needed phylogenetic framework for future research on the genus.

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Appendix I

Experimental assessment of mating opportunities in wild bird populations

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Abstract

Mating opportunities may differ between closely related species, although the evidence for such variation is scant. Here we use an experimental approach to estimate mating opportunities in wild bird populations. By experimentally creating unmated males and females, we show that mating opportunities are different between closely related plover species (*Charadrius spp.*): mating opportunities are male-biased in the Kittlitz's plover but unbiased in white-fronted plovers. The strength of experimentally induced pair bonds also differed between the two species: newly established pair bonds were dissolved and new mates replaced by original mates in 12 out of 12 white fronted plover pairs, whereas none of the 16 newly formed pair bonds was dissolved in the Kittlitz's plover ($P < 0.001$). Taken together, these results are important in highlighting interspecific variation in mating activities, as indicated by the operational sex ratio (OSR), and pair bond stability. These variations in turn, may influence mating systems and parental care.

Author contributions
JEP: field work, sample acquisition, statistical analysis, manuscript preparation
MB: field work, sample acquisition, manuscript improvement
NdR: molecular sex-typing, manuscript improvement
TS: statistical advice, manuscript improvement

This work was conducted as part the PhD research of JEP.

Introduction

The differing evolutionary interests of males and females over reproduction (termed sexual conflict, Parker 1979) are a pervasive evolutionary force influencing the behaviour, ecology and life histories of many organisms (Chapman *et al.* 2003; Arnqvist & Rowe 2005). A common issue when the interests of males and females are antagonistic concerns offspring care (Trivers 1972; Maynard Smith 1977; Houston *et al.* 2005; Lessells 2012). By caring for the offspring, parents often improve the growth and survival of the young (Clutton-Brock 1991; Klug *et al.* 2012); for example, by gestating, nursing, protecting and feeding the young, the offspring have improved chances of survival (Tyler *et al.* 1983; Balshine-Earn & Earn 1998; Baeza & Fernández 2002; Klug *et al.* 2012). However, care is costly in terms of time and energy, and the caring parent can be killed by predators (Veasey *et al.* 2001; Li & Jackson 2003; Klug *et al.* 2012). Therefore, whilst both biological parents benefit from providing care for the offspring, each parent is expected to withhold his (or her) parental contribution in order to raise further offspring in future (Houston *et al.* 2005; McGraw *et al.* 2010; Lessells 2012).

Theory suggests that a key component of conflict resolution between male and female parents is mating opportunity (Székely *et al.* 2000; McNamara *et al.* 2000; Kokko & Jennions 2008; Klug *et al.* 2012). If one sex has more favourable mating opportunities than the other, the former parent is expected to reduce (or completely terminate) care more often that its mate, and seek out a new partner (Székely *et al.* 1996; Balshine-Earn & Earn 1998; Pilastro *et al.* 2001). Mating opportunities can be assessed by estimating the ratio of sexually active males to females (operational sex ratio, Kvarnemo & Ahnesjö 1996; Forsgren *et al.* 2004). An alternative approach to estimating mating opportunities is to experimentally create unmated individuals, and to quantify their re-mating behaviour, e.g. time to re-mate, re-mating success and reproductive success with the new mate. This experimental approach is powerful, since it directly assesses the mating potential of unmated individuals at a given time in a population.

Here we compare mating opportunities between three closely related plover species: the Kentish plover (*Charadrius alexandrinus*), white-fronted plover (*C. marginatus*) and the Kittlitz´s plover (*C. pecuarius*). Small plovers (*Charadrius spp.*) exhibit substantial variation in their breeding systems, since some of these species are monogamous and both parents rear the young, whereas others exhibit male and/ or female polygamy and a single parent (the male or the female) raises the young to independence (Székely *et al.* 2006; Thomas *et al.* 2007). In addition, plovers typically

breed in open areas, and their nests and broods are therefore accessible for experimental manipulations (Székely & Cuthill 2000).

A previous experiment established that mating opportunities were female-biased in the Kentish plover (Székely *et al.* 1999), and this result was consistent with demographic analyses that estimated about 6 times more adult males than females in the population (Kosztolányi *et al.* 2011). Skewed adult sex ratios (ASRs) are common in wild populations (Donald 2007), and recent work suggests that biased ASRs predict sex roles (Liker *et al.* 2013). Here we use identical experimental protocol in two close relatives of the Kentish plover, the white-fronted plover and Kittlitz's plover, to compare mating opportunities between these three plover species. All three species are insectivorous ground-nesting birds that exhibit similar life-histories and ecology (adult body masses, Kentish plover: 41.8 g; white-fronted plover: 37.1 g; Kittlitz's plover: 35.3 g, Urban *et al.* 1986; Hockey *et al.* 2005). The latter two species are common breeding birds in Africa, and their parental care systems differ from the Kentish plover that exhibits male-biased parental care (Székely & Lessells 1993): white-fronted plovers have biparental care, whereas Kittlitz's plovers are reported to have uniparental care, carried out by either the male or the female parent (Tree 1974; Urban *et al.* 1986; Hockey *et al.* 2005).

Based on theoretical models (Kokko & Jennions 2008; Klug *et al.* 2012) and information on parental care systems, we derived three predictions. First, we predicted higher mating opportunities in uniparental species (Kittlitz´s plover) than in biparental species (white-fronted plover), since in biparental species both parents are engaged with care until the offspring are fully independent (henceforth, between-species hypothesis). Second, we predicted no difference in mating opportunities between males and females in biparental species given that both sexes are fully engaged in parental care. Similarly, no difference in mating opportunities between males and females was predicted for the uniparental species in which either parent is free to seek a new mate (henceforth, between sexes hypothesis). Third, we predicted intense courtship behaviour by males and females both in biparental white-fronted plover and uniparental Kittlitz´s plover where care is provided by either parent (henceforth, courtship hypothesis). We include the Kentish plover in our analyses (using the data from Székely *et al.* 1999), since the same experimental methodology was used in all three experiments. Nevertheless, our main conclusions remain consistent by restricting the analyses to the white-fronted and Kittlitz´s plovers.

Methods

Study Species and Study Sites

White-fronted plovers and the Kittlitz's plover were investigated in SW Madagascar (for Kentish plover, see details in Székely *et al.* 1999). Kittlitz's plovers were studied between 6 February 2010 and 13 May 2010 in Andavadoaka (22° 02' S, 43° 39' E) where they breed around alkaline lakes. Approximately 300 Kittlitz's plovers breed in Andavadoaka (Parra, Zefania and Székely unpubl. data). Fieldwork with the white-fronted plover was carried out between 1 April 2011 and 23 June 2011 at Lake Tsimanampetsotsa National Park (24° 3' S, 43°44' E), a large alkaline lake (approx 15 km x 0.5 km), surrounded by sandy shores, short grass and saltpans. Approximately 150 white-fronted plovers breed around the lake (Parra, Zefania and Székely unpubl. data). We captured 18 Kittlitz's plover pairs (36 individuals) and 14 white-fronted plover pairs (28 individuals) in total. Morphological traits (body mass, tarsus length, wing length and bill length) were measured using a spring balance, a sliding calliper and wing ruler (see details in Kentish plover field guide, www.bath.ac.uk/bio-sci/biodiversity-lab/pdfs/KP_Field_Guide_v3.pdf). All adults were ringed with an individual combination of colour rings and a numbered SAFRING metal ring from the University of Cape Town, South Africa.

Molecular Sexing

Both plover species have sexually monomorphic plumage (Urban *et al.* 1986; Hockey *et al.* 2005), therefore we used molecular sex-typing to determine the sex of individuals (dos Remedios *et al.* 2010). A small blood sample was taken from each adult's brachial wing vein, by puncturing, collecting drops of blood (~25 ul) in capillary tubes, and storing this in Eppendorf tubes of Queen's Lysis Buffer. DNA was extracted from blood samples using the Ammonium Acetate extraction method (Nicholls *et al.* 2000; Richardson et al. 2001). For molecular sex-typing, Z- and W-chromosome specific genes were amplified via polymerase chain reaction (PCR) using the Z-002B/Z-002D primers (Dawson 2007). For additional certainty in sex assignment, the W-chromosome specific Calex-31 primers, developed in the genus *Charadrius* were utilized (Küpper *et al.* 2006). PCR amplification was conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler under the following conditions: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 56°C for 90 s, 72°C for 60 s with a final extension of 60°C for 30 min. Samples were visualized on an ABI 3730 automated sequencer. IR Dye-labelled tailed primers separated the products of Z-002B/Z-002D primers into either one (ZZ) or two bands (ZW), indicating male or female respectively. The W-specific Calex-31 product appeared

as one band indicating female only. Images were scored using GeneMapper software version 4.1 (Applied Biosystems). To maximize reliability, all samples were sexed using two markers. Additionally, for 8% of samples (11 Kittlitz's plover and 10 white-fronted plover individuals) molecular sexing was repeated; in all cases, repetitions concurred with original results.

Experimental Manipulation

We used the methodology developed by Székely *et al.* (1999). Briefly, both parents were caught on the nest, and a random parent (male or female) was released immediately after ringing, measuring and blood sampling. The other parent was taken into captivity (see below). Only pairs incubating two eggs (modal clutch size in both species) were manipulated. Egg length and breadth were measured with a sliding calliper, and the number of days eggs had been incubated for was estimated based on the floatation stage of the egg in a transparent jar with clean water (see details in Kentish plover field guide). Eggs were distributed to other non-experimental plover clutches at approximately the same stage of incubation.

Removed plovers were transported to a purpose-built aviary near the field camp at both study sites. The aviaries had four units for Kittlitz's plovers and six units for white-fronted plovers. Each unit consisted of a 1 m x 1.5 m x 1.5 m (height x length x width) wood frame fitted with chicken mesh (13 mm x 13 mm). To provide shade for the captive birds, we covered the outside of the aviary with papyrus, *Cyperius sp.,* and fitted 50 cm of cloth at the base of the mesh inside the units. Captive plovers were fed with appropriate food and drink three times per day every day to maintain their good health using high protein meals: dried insects for wild birds (dried mealworms, dried earthworms, shrimps and dried waterfly; shop.naturesgrub.co.uk/), bird supplement vitamins (Vitacombex V; www.petland.co.uk) and pinhead oatmeal (Prosecto InsectivorousTM; www.haiths.com). Captive plovers were also supplied with fresh beetles twice a day using pit fall traps set-up in the salt-marsh. Captive plovers were released after their former mate found a new mate (or their former mate was not seen for at least 12 days), and were measured and ringed before release. Captive plovers were in good body condition at release, since many remated shortly after release (see Results).

Behavioural Observations

The released plovers were searched for every day in the field using a car and mobile hide (Székely *et al.* 2003). When a plover was found; instantaneous samples were recorded for 30 min at 30 seconds intervals. Attempts were made to record the behaviour of focal plovers on at least two occasions before they found a new mate. Behavioural categories developed for the Kentish plover (Székely *et al.* 1999) were used for both Kittlitz's and white-plovers; two observers recorded all field observations (M. Beltrán and J.E. Parra). Mated individuals were identified by courting behaviours such as scrape ceremony, nest-scrape and copulation (Urban *et al.* 1986; Hockey *et al.* 2005, see Supplementary movies courting and copulation of Kittlitz´s plover). New pairs were checked for clutches every day, and eggs were measured as described above.

Data Processing and Statistical Analyses

Date of mating was defined as the mean date between the date when a plover was last seen single and the first date it was seen with a new mate. Mating time was the difference between date of release (either on the day of manipulation or from captivity) and date of mating. The response variable, mating time, was analyzed using generalized linear models (GLM) with Tweedie (1.5) error structure and a log link function. The models investigated the effects of two main variables: species (Kentish, white-fronted and Kittlitz's plovers) and sex; and three additional fixed variables: type of manipulation (released in the field or from captivity), release date, and number of days in captivity (see Table 1). Dates were expressed in Julian date format, i.e. number of days since 1 January. Akaike's information criterion for small sample sizes (AICc) is presented for GLM models where lowest AICc score is the best supported model (Symonds & Moussalli 2010, Table 1).

Mating time was also analyzed using survival analyses and these estimates are referred to as expected mating times. In these analyses, the terminal event (outcome) was the occurrence of mating, defined as the first observation when a plover was seen with a mate (see Székely *et al.* 1999). Several individuals did not find a new mate when we saw them for the last time, and these were treated as censored observations. First, we used a Gehan-Wilcoxon test to compare expected mating times curves (survival curves) of species of plover by sex. Survival curves were generated by the Kaplan-Meier method. Second, for each plover species a separate Cox regression model was constructed to investigate the probability of remaining single from the day of release (season), sex and their interaction as covariates (Table 2).

Courting behaviour was measured as the percentage obtained from each 30 minutes sample. For individuals with several behavioural observations, we calculated the mean percentage of courting. Courting behaviour was analyzed using GLMs with Tweedie (1.5) error structure and a logarithmic link function per individual plover. The model included two main factors: species of plover and sex; and three additional fixed variables: type of manipulation (released in the field or from captivity), released date, and number of days in captivity. Model selection and statistical parameters estimated for each independent variable in the models are provided in the Appendix, Table A1. In addition, for each sex a separate GLM model was constructed to investigate the effect of three species of plovers on courting behaviour (Table 3). Data were analyzed by using SPSS statistics for Windows version 19 and figures were made in R (R Development Core Team 2008) using the package ggplot2 (Wickham 2009).

Ethical Note

The experiments in Madagascar were approved by the Ministry of Environment, Forests and Tourism of the Republic of Madagascar (Research permit No: 053/11/MEF/SG/DGF/DCB.SAP/SCB of 11 March 2011 and 132/10/MEF/SG/DGF/DCB.SAP/SSE of 06 May 2010) and Madagascar National Parks (No: 398-10/MEF/SG/DGF/DVRN/SGFF of 18 May 2011). The Kentish plover experiment was approved by the Turkish Ministry of Environment (see Székely *et al.* 1999). Captive plovers were monitored daily and kept in standard conditions (see Experimental Manipulation) to reduce their stress levels.

Results

Between Species Hypothesis

Mating opportunities differed significantly between the three plover species (Fig. 1): white-fronted plovers mated significantly more quickly (median = 2.0 days, range 0.5 – 4.5 days, $N = 12$) than both Kittlitz's plovers (median = 5.1 days, range 1.0 – 11.5 days, $N = 16$) and Kentish plovers (median = 6.3 days, range 0.5 – 47.5 days, $N = 34$; GLM: $\chi^2_2 = 11.59$, $P = 0.003$, Table 1).

These results remained consistent using survival analyses that also included the individuals that were not successful in finding a new mate (Fig. 2, Table 2; see Methods). The proportion of plovers remaining single was significantly lower for the white-fronted plover (median = 4 days, $N = 14$) than both the Kittlitz's plover (median = 14.6 days, $N =$

33) and Kentish plover (median = 13.4 days, *N* = 59; testing the three species, Gehan-Wilcoxon test= 16.316, *P* < 0.001).

Between Sexes Hypothesis

A significant species by sex interaction suggested a sex-based difference in mating opportunities (GLM: χ^2_2 = 47.62, *P* < 0.001, Table 1). Female Kittlitz's plovers took significantly longer to mate (median = 6.5 days, range 3.5 – 11.5 days, *N* = 6) than males (median = 3.3, range 1.0 – 7.5 days, *N* = 10; least significant difference test (LSD): *P* = 0.047) whereas the opposite was found in the Kentish plover (Székely *et al.* 1999). However, male and female mating times were not significantly different in white-fronted plovers (male: median: 2.0 days, range 0.5 – 3.5 days, *N* = 6; female: median: 2.0 days, 1.0 – 4.5 days, *N* = 6, LSD-test: *P* = 0.823).

Figure 1. Mating times in three species of plovers. The lower and upper borders of the box are lower and upper quartiles, respectively, the horizontal bar is the median and whiskers represent the lowest and highest observations.

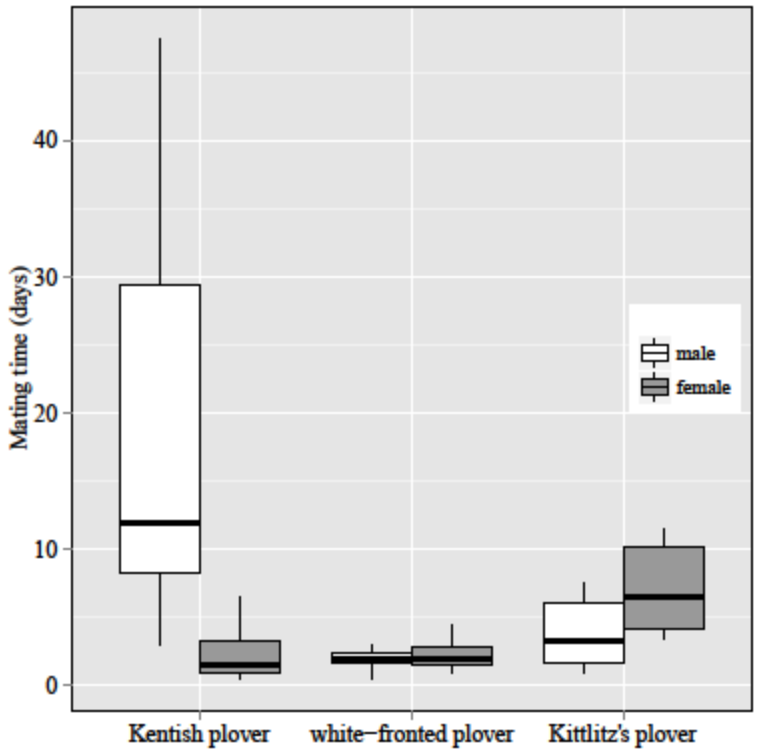

Table 1. Mating times (response variable) of males and females in three species of plover. GLMs were used to analyze mating time using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc).

Independent variable	Full model (AICc = 346.004)			Best model (AICc = 341.088)		
	Wald χ^2	*df*	*P*	Wald χ^2	*Df*	*P*
(Intercept)	38.596	1	<0.001	49.365	1	<0.001
Between species prediction						
Species	11.248	2	0.004	11.595	2	0.003
Between sexes prediction						
Sex	4.072	1	0.044	3.974	1	0.046
Species * sex	39.65	2	<0.001	47.620	2	<0.001
Manipulation	0.290	1	0.59	-	-	-
Release date	4.818	1	0.028	5.007	1	0.025
Captive days	0.646	1	0.422	-	-	-

Figure 2. Proportion of males and females remaining single in three species of plovers: Kentish plover (top), white-fronted plover (middle) and Kittlitz's plover (bottom). Dotted lines show the median expected mating time of males and females after release. Number of individuals: 32 male and 27 female Kentish plovers; 7 male and 7 female white-fronted plovers; and 17 male and 16 female Kittlitz´s plovers.

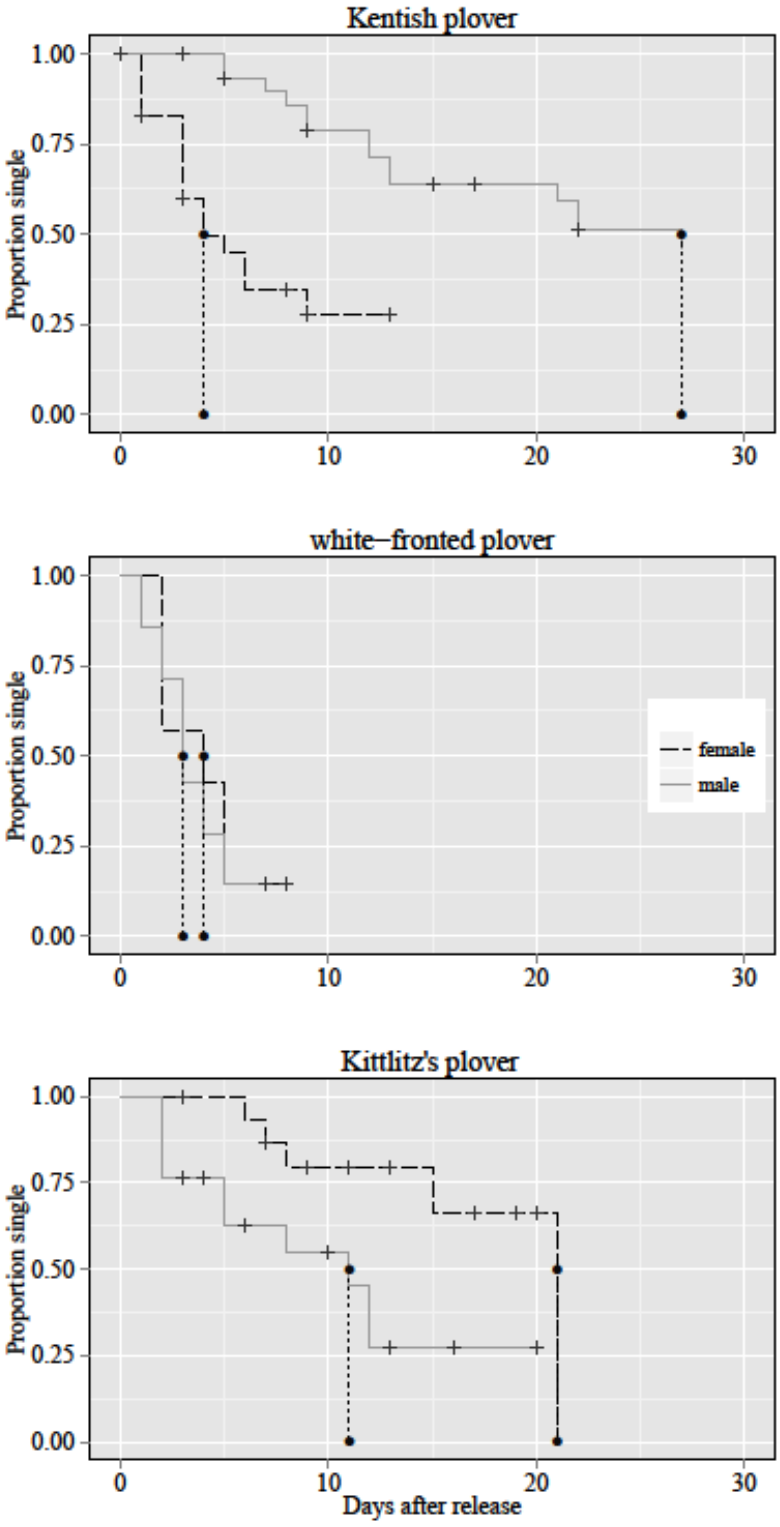

Table 2. Mating time in plovers using Cox proportional hazard models. To examine the relationship of the survival distribution, which includes censored observations, with the covariates sex and release day. For each species a separate model was constructed. Number of individual Kentish plovers, mated = 34, censored = 19; white-fronted plover, 12, 2; Kittlitz's plover, 16, 17, respectively.

Species	Variable	B	Wald χ^2	*df*	p
Kentish plover	Sex	1.541	12.07	1	0.001
	Release date	-0.024	6.073	1	0.014
White-fronted plover	Sex	-0.18	0.083	1	0.77
	Release date	0.002	0.004	1	0.95
Kittlitz's plover	Sex	-1.342	4.864	1	0.027
	Release date	-0.01	0.088	1	0.767

Figure 3. Courtship behaviour in three species of plovers. The lower and upper borders of the box are lower and upper quartiles, respectively, the horizontal bar is the median and whiskers represent the lowest and highest observations. Circles denote outliers that are between the first and third interquartile from the nearer edge of the box.

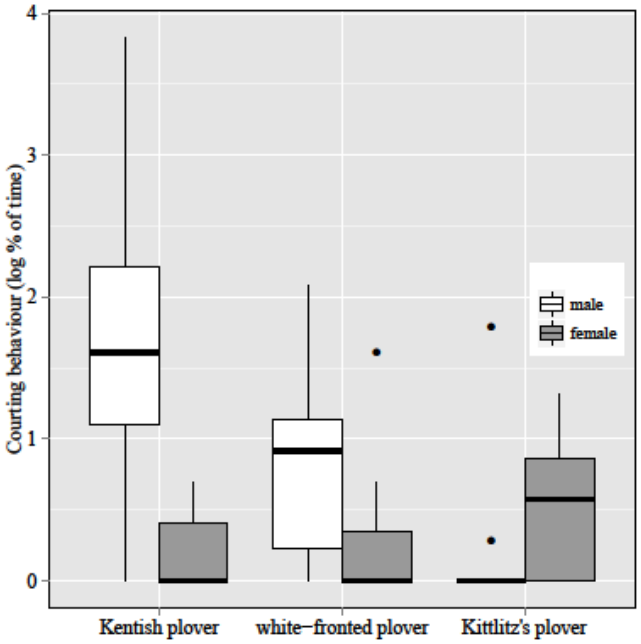

Table 3. Courtship behaviour (response variable: % of time courting) in three plover species. GLMs were used to analyse percentage of time courting using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc).

Sex	Variables	Wald χ^2	*df*	*P*
Male	(Intercept)	13.176	1	< 0.001
	Species	10.689	2	0.005
Female	(Intercept)	0.155	1	0.694
	Species	1.437	2	0.487

These results remained consistent using survival analyses (Table 2): the proportion of female Kittlitz's plovers remaining single was higher than that of males (male median: 11.0 days, $N = 17$, female median: 21.0 days, $N = 16$, Fig. 2), whereas the proportion of single males and females were not significantly different in the white-fronted plover (male median: 3.0 days, $N = 7$, female median: 4.0 days, $N = 7$, Fig. 2).

Mating time increased over the season only for the female in the Kentish plover (Cox regression: $\chi^2_1 = 7.66$, $P = 0.014$), suggesting an influence of time of breeding season on mating opportunities in the Kentish plover, although this was not the case in the other two species (Table 2).

Courtship Hypothesis

Courtship behaviour had a significant species by sex interaction (GLM: $\chi^2_2 = 6.329$, $P = 0.042$, Appendix: Table A1, Fig. 3). Courtship behaviour by males differed significantly between plover species (GLM: $\chi^2_2 = 10.689$, $P = 0.005$, Table 3), whereas courtship behaviour by females did not differ between species (GLM: $\chi^2_2 = 1.437$, $P = 0.487$, Table 3). In contrast to the Kentish plover, which exhibited male-biased courtship behaviour (LSD-test between male and female Kentish plovers: $P = 0.005$), males and females of the other two species spent comparable times on courtship (white-fronted plover: LSD-test: $P = 0.252$; Kittlitz's plover: LSD-test: $P = 0.679$, Fig. 3).

Pair Bonds

The new pair bonds in experimentally-induced white-fronted plovers were significantly weaker than in Kittlitz's plover: in 12 white-fronted plovers that mated after their

former partner was removed (6 males, 6 females), all experimentally-induced pair bonds were replaced by the original mates after they were released from captivity. In contrast, in 16 Kittlitz's plovers that mated after their former partner was removed (10 male and 6 female), none were replaced by their former mates once their former mates were released from captivity (Fisher's exact test comparing the frequencies of mate replacement (Y/N) between white-fronted plover and Kittlitz's plover: $P < 0.001$).

Discussion

These experiments provided four key results. First, they show that mating opportunities are significantly different between closely related species. This result is striking because two of these plover species (white-fronted and Kittlitz's) breed in the same habitat in Madagascar, and therefore, ecological explanations are unlikely to explain the differences in mating opportunities. The rapid mating of white-fronted plovers – a pattern we did not expect – suggests that there is a large pool of floating individuals that can rapidly move in to pair up with unmated individuals. Consistent with this argument, both male and female white-fronted plovers stayed in the same territory, and new individuals moved in to replace the removed mates.

Second, we found sex-bias in mating opportunities: the male-biased mating opportunities in Kittlitz's plover were the opposite of those found in the Kentish plover, whereas in white-fronted plovers mating opportunities did not differ between males and females. As far as we are aware, our study is the first to experimentally demonstrate differences in sex-biased mating opportunities between closely related species. Sex-biased mating opportunities may emerge in two ways. One explanation is that the ratio of sexually active males to females (operational sex ratio, OSR) may not be at parity. OSR may be biased due to differences in the reproductive schedules of males and females ("time in", "time out"), or biased adult sex ratios (ASR, Donald 2007, Székely *et al.* submitted). Evidence suggests that OSR can vary due to mating and parental care activities (Forsgren *et al.* 2004, Symons *et al.* 2011; LaBarbera *et al.* 2011; Canal *et al.* 2012). In addition, recent studies found substantial difference in ASR between closely related species (Liker *et al.* 2013). Further works are needed to separate whether biased mating opportunities emerge via different reproductive scheduling or biased ASR in plovers. For one of these species, the Kentish plover, a demographic study confirmed male-biased ASR (Kosztolányi *et al.* 2011), although ASR has not been estimated for the white-fronted and the Kittlitz's plover. Alternatively, sex-biased mating opportunities may arise due to differences in the willingness of males and females to re-mate. For

instance, the post-breeding refrectory periods may differ between males and females (Cantoni & Brown 1997; Balshine-Earn & Earn 1998): females typically need more time to recover than do males. However, the latter explanation is unlikely, since the adult plovers used in the plover experiments had breeding efforts interrupted and sought new mates shortly after removal of their original mate (or on release from captivity). Furthermore, several female Kentish plovers remated within less than a day – a pattern that is inconsistent with the explanation that females need more time to recover than do males.

Third, male courtship behaviour was different between the three species: male Kentish plovers spent more time on courtship than male white-fronted and Kittlitz's plovers. This pattern is consistent with the explanation that ASR is male-biased in the Kentish plover. The significance of this result is that courtship behaviour is variable between closely related species and suggests that ASR, and in turn the OSR, is related to the intensity of male mating competition. Consequently, comparable intensity of courtship behaviour was observed between sexes in the Kittlitz´s and white-fronted plovers implying that males and females may compete similarly for available mates; regardless of conventional sex roles: male-male competition and female choice for mates (Vincent *et al.* 1994). In addition, variation in critical resources for breeding has been shown to change the OSR, and then the intensity of competition for mates (Forsgren *et al.* 1996). Availability of breeding territories, for example, may affect the OSR, since the sex that holds the territories will be limited by scarcity of nest sites. In a sand goby population (*Pomatoschistus minutes*), for instance, nest-site abundance can influence the intensity of male mating competition (Forsgren *et al.* 1996). Hence, the dynamic of OSR, and in turn mating competition, is probably modulated by both ASR and resource availability(Kvarnemo & Ahnesjö 1996, Forsgren *et al.* 2004).

Finally, the new pair bonds were significantly weaker in white-fronted plovers than in Kittlitz's plover suggesting that the former species exhibits long-term pair bonds whereas the latter has short-term pair bonds. Mate fidelity may emerge in two ways. On the one hand, former mates may actively seek out each other *per se*, and prefer to mate with each other. On the other hand, mate fidelity may emerge via site fidelity: white-fronted plovers are highly territorial (Lloyd 2008), and therefore upon release from captivity, individuals return to their former territories and chase out their former partner's new mates. Established pairs may prefer to reunite because of the fitness benefits in terms of synchronisation of behavioural and physiological characteristics such as defence of breeding territories, courtship behaviours, laying date, incubation,

chick-raising between others which have been shown to improve with time and experience of the pair (Rowley 1983; Bried *et al.* 2003). In the black turnstone *Arenaria melanocephala*, for example, higher production of offspring was reported for established pairs in contrast to new pairs formed by the divorce or death of a mate (Handel & Gill 2000). Improved breeding with increased experience is also well known in geese, albatross and other long-term pair bonding animals (Black 2001; Angelier *et al.* 2006).This is significant because mate fidelity and site fidelity may influence the OSR and the intensity of mating competition, since limited breeding vacancies might limit the reproduction of sexually-active floater individuals, increasing intrasexual competition in a comparable way for males and females.

In conclusion, using an experimental approach we found significant differences in the mating opportunities of closely related plover species. As mating opportunity is linked to OSR and ASR, our work suggests that substantial variation in OSR (and possibly ASR) is exhibited among closely related species. Such variation may influence the direction and intensity of competition in males and females for mates and breeding territories. These differences in OSR, in turn, may facilitate different intensities of sexual selection and induce different mating systems and patterns of parental care.

Acknowledgments

We thank Tody Tsiry for assistance with field work, and the comments of Hanna Kokko and Tamás Székely Jr on former versions of the manuscript. This study is part of the PhD dissertation of J.E. Parra, and was funded by University of Bath Excellence Studentship and the Leverhulme Trust (ID200660763, Ecology and genetics of endemism) to T. Székely. Molecular sexing was supported by NERC-Biomolecular Analysis Facility at the University of Sheffield.

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Appendix II

Sex differences in incubation behaviour but not mortality risk in a threatened shorebird

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Abstract

Sex-related variation in survival is common in birds and, as it influences effective population size and population growth, is important for conservation and species management. Here we assessed incubation behaviour and sex-related survival in a threatened sexually monomorphic shorebird, the St Helena Plover *Charadrius sanctaehelenae*. Males incubated at night, the period of highest activity of cats, which are likely to be predators of breeding birds. In spite of behavioural differences between the sexes, adult survival was not significantly different between males and females, suggesting that sex-biased behaviour need not lead to sex-related survival, and thus behavioural differences may not impact upon the adult sex ratio.

Published, 2013: Ibis 155: 877-880.

Author contributions

FB: field work, sample acquisition, statistical analysis, manuscript preparation
NM: manuscript improvement
NdR: molecular sex-typing, manuscript improvement
MB: manuscript improvement
TS: statistical advice, manuscript improvement

This work was conducted as part of the PhD research of FB.

Introduction

Adult sex ratio (ASR) is one of the key demographic traits influencing population dynamics (Lee *et al.* 2011). ASR is usually skewed toward males in birds and is primarily attributable to higher adult female mortality (Donald 2007). Understanding patterns of, and reasons underlying, ASR and sex-related demography can inform species conservation (e.g. Clout *et al.* 2002). Differential survival can influence the effective population size and therefore the population growth rate. Gruebler *et al.* (2008) found that a population of Eurasian Whinchats *Saxicola rubetra* was declining 1.7 times faster than anticipated because adult mortality was differentially affecting incubating females. Furthermore, a skewed ASR may affect a population's ability to recover, in particular if males are over-represented (Bessa-Gomes *et al.* 2004). Donald (2007) found that threatened birds consistently exhibited more male-biased ASRs than nonthreatened ones.

Here we investigate parental behaviour in an endangered shorebird, the St. Helena Plover *Charadrius sanctaehelenae* (BirdLife & International 2009, McCulloch 2009), endemic to the Atlantic island of St Helena, to assess whether behavioural differences in incubation are associated with sex-related adult survival. Previously we showed that clutch survival is low (average clutch survival = 21.5%) and that the majority (85%) of nest predation occurs at night. The most common predator recorded was the domestic cat *Felis catus*, followed by rats *Rattus norvegicus* or *Rattus rattus*, and Common Myna *Acridotheres tristis* (Burns 2011).

Although direct evidence is scarce for other life stages, it is likely the same species prey upon chicks and that cats are the main predator of adults (McCulloch 2009). As cats are mostly active at night, we conjectured that this may lead to reduced survival of the sex that incubates at night. If, like many other threatened bird species, survival is sex-related in the St. Helena Plover, increasing predation pressure may have a compound effect on the species' persistence. Investigating sex-related differences in behaviour and mortality, however, is challenging in sexually monomorphic species such as the St Helena Plover. We therefore used molecular markers (Ellegren 1996, Griffiths *et al.* 1998, dos Remedios *et al.* 2010) to sex adult and young St Helena Plovers.

The aim of this study is to assess whether incubation patterns or survival are sex-related in the St. Helena Plover. If there is a bias towards one sex undertaking the majority of nocturnal incubation, we predict that its survival may be suppressed.

Methods

On St Helena (15°58`S, 5°43`W) data were collected at the main breeding sites: Deadwood Plain, Broad Bottom, Man and Horse, Upper Prosperous Bay and Prosperous Bay Plain (locations shown in McCulloch 2009). The first three sites are pasture areas and the others semi-desert. Fieldwork was carried out during 2007–2010 in three field seasons, the first two November to February, and the third January to February. Birds were ringed during the first two field seasons (Burns *et al.* 2010).

Trapping was done during daylight. Chicks were caught by hand, measured and given a BTO metal ring at hatching and individually colour-ringed at 2 weeks. During the second field season blood samples, c.25 µL, were taken for molecular sexing. Molecular sexing using faecal samples from field season one was unsuccessful, as the volume of DNA extracted was insufficient for analysis.

Data from 34 juveniles (30 genetically sexed as chicks and four by mating behaviour when adult) and 64 adults were used in our analysis. Of the adults, 39 were sexed using blood samples and 35 from mating behaviour or having a known sex mate. Sex of all individuals that were sexed using both methods agreed (n = 10).

DNA was extracted using the ammonium acetate method (Nicholls *et al.* 2000; Richardson et al. 2001). CHD-Z and CHD-W genes were amplified via PCR using the P2/P8 primers (Griffiths *et al.* 1998). For additional certainty in sex assignment, the Z-002B/Z-002D primers (Dawson *et al.* unpubl. data) were used. IR dye-labelled tailed primers separated the product of Z-002B/Z-002D and P2/P8 primers into one (ZZ) or two bands (ZW), indicating male or female, respectively.

Incubation behaviour was scored using footage from small (2.5-cm diameter), motion-sensitive cameras with infrared light arrays (male, female or neither on the nest) (Bolton *et al.* 2007). Activities of less than 10s duration were not scored. Seven 24-h periods were recorded from five nests where the sexes could be identified. The recordings are from nests of different ages, but all had a complete clutch of two eggs (modal clutch size) prior to camera installation. A mixed model with binomial errors was used to investigate the balance of female to male incubation throughout the day. A categorical factor with two levels that differentiated between night and day was used as an explanatory variable, and nest identity and 24-h period were used as random factors. The binomial denominator was total incubation duration (minutes) and the two-vector response variable, signifying the total incubation duration for females and males, estimated separately for day and night.

The analysis was done using PROGRAMME R (R Core Development & Team 2004). Apparent survival (Φ) and resighting probability (p) for adults and juveniles

were estimated using MARK (White & Burnham 1999). The complement of apparent survival is mortality plus permanent emigration. For St Helena Plovers the latter will be small as they do not disperse from the island and resighting efforts covered all known sites. Resighting was carried out on five occasions: November 2008, February 2009, August 2009, October 2009 and January–February 2010. The final resighting period was a year after ringing stopped, at which time all birds ringed as chicks would have reached adulthood. Only juveniles known to have fledged were included, to differentiate between chick and juvenile survival, the latter taken as survival from fledging to 1 year. Due to the short duration of the study, temporal variation in survival was not investigated.

Analysis included known-sex birds only and all birds aged 1 or older were classed as adults, juveniles changing category to become adults upon reaching 1 year old. Adults and juveniles were treated separately in the analysis, as survival is generally substantially lower for young animals prior to reaching sexual maturity. The general model was *Φ (sex) p (sex),* for adults and juveniles. Goodness-of-fit testing used median ĉ and models were adjusted accordingly. First, we compared a series of models with different recapture terms, testing for temporal variation in recapture or sex-related variation in recapture for both adults and juveniles. The most parsimonious model was selected as that with the lowest AIC. The most parsimonious recapture term was then used in a second series of models investigating sex-related survival, again using AIC to select the most parsimonious model.

Results

Nests were incubated 77% of the time and an interceptonly version of the mixed model showed that male and female incubation duration did not differ over the 24-h period ($t_7 = 0.309$, $P = 0.766$; 0.48 of total incubation by females; Fig. 1). The balance of incubation between males and females was significantly different between the daylight period (sunrise 05:56h to sunset 18:55h) and at night (logistic mixed model, $t_6 = 3.367$, $P = 0.015$; Fig. 1). During daylight, females incubated more than males (65% of total incubation), whereas they incubated far less during the night (24% of total incubation).

There was no support for sex-related survival or resighting for adults or juveniles (Table 1, monthly adult survival (Φ_A) = 0.977 (95%CL: 0.966–0.985), annual Φ_A = 0.758 (0.656–0.833), monthly juvenile survival (Φ_J) = 0.940 (0.895–0.966), fledging to 1-year Φ_J = 0.536 (0.328–0.708)). Birds of known sex make up only a subset of birds ringed (around 60% of ringed adults are of known sex). Equivalent values for the full dataset, including unsexed birds, give annual Φ_A = 0.829 (0.748–0.887) and fledging to

1-year Φ_J = 0.471 (0.325–0.606) (Burns *et al.* 2013). Burnham and Anderson (2002) suggest that models within two Akaike information criterion (AIC) units of the minimum should be considered for model averaging. The difference between the top and second placed model, which includes sex-related adult survival, is 1.34 AIC units. Nevertheless, the 95%CL for female and male survival in the latter
model are largely overlapping, lending little support for sex-related survival (monthly ♀ Φ_A = 0.981 (0.965–0.989), monthly ♂Φ_A = 0.973 (0.953–0.984)).

Figure 1. Proportion of incubation by female (dark grey) and male (white) St Helena Plovers. Light grey indicates that neither parent is on the nest, n is given above each bar.

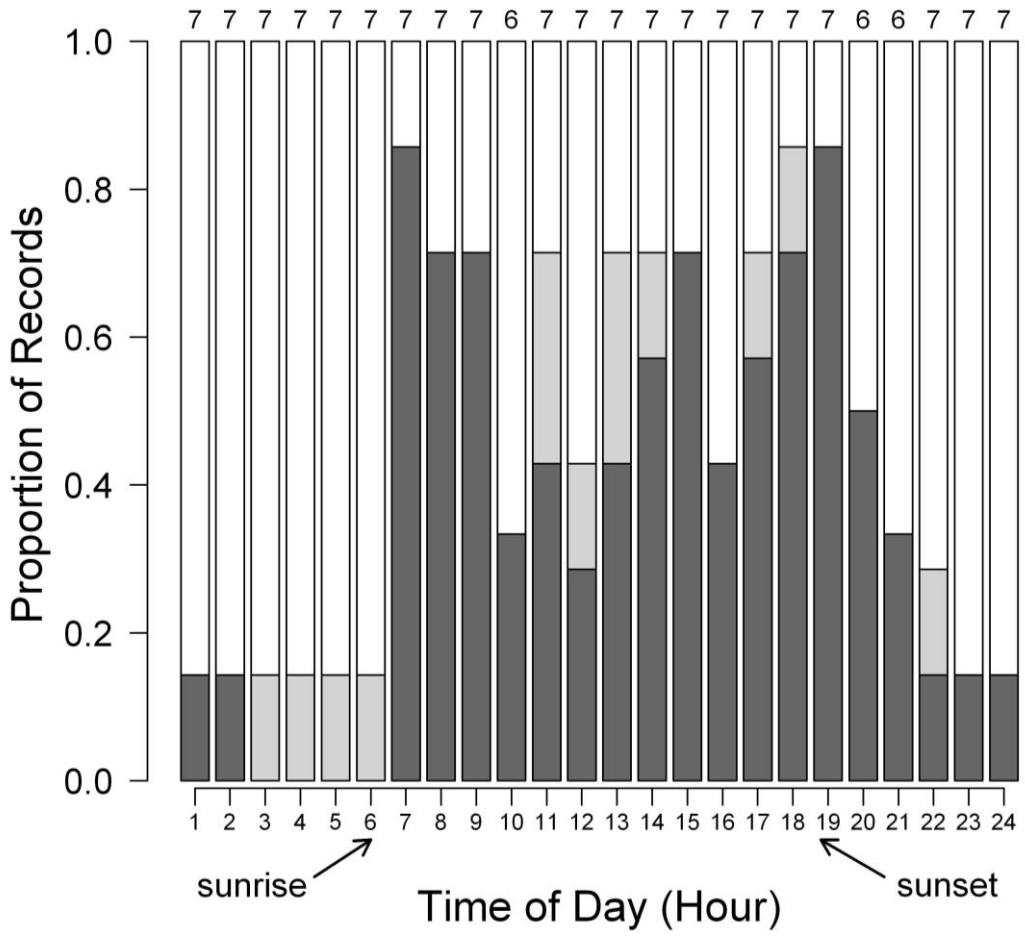

Table 1. Survival analysis of adult (A) and juvenile (J) St Helena Plover using MARK.

Model specification		n = 64_A (26_M38_F), 34_J (17_M17_F)			
Survival	Resighting	ΔQAIC	K	Deviance	Weight

$\Phi_{A,}\Phi_J$	P_A, P_J[a]	0	4	343.870	0.413
Φ_A (sex), Φ_J	P_A, P_J	1.337	5	343.145	0.212
Φ_A, Φ_J (sex)	P_A, P_J	1.572	5	343.380	0.188
Φ_A (sex), Φ_J (sex)	P_A, P_J	2.903	6	342.638	0.097
Φ_A (sex), Φ_J (sex)	P_A, P_J (sex)	4.773	7	342.422	0.038
Φ_A (sex), Φ_J (sex)	P_A (sex), P_J	5.232	7	342.880	0.030
Φ_A (sex), Φ_J (sex)	P_A (sex), P_J(sex)	7.080	8	342.629	0.012
Φ_A (sex), Φ_J (sex)	P (time)	7.347	9	340.784	0.010

Discussion

The survival of male St Helena Plovers could be compromised if they incubate largely at night, like males of several other *Charadrius* species (Kosztolányi & Székely 2002, St Clair *et al.* 2010), as this is the active period of most predators of breeding plovers on St Helena. Amat and Masero (2004) found male Kentish Plovers *Charadrius alexandrinus*, which show similar partitioning of incubation, were more likely to be predated at the nest than females. They attributed this to lower visibility at night, and therefore poorer escape responses.

Additionally, it has been suggested that the female-biased sex ratio of the Stewart Island population of the New Zealand Dotterel *Charadrius obscurus* developed due to lower male survival during night time incubation (Dowding *et al.* 1999). Whether males or females are at greater risk will depend, however, on the local predator community. For instance, Two-banded Plovers *Charadrius falklandicus* show a similar incubation pattern but females may be at greater risk, as all nest predations were filmed during daylight (St Clair *et al.* 2010).

Despite several comprehensive studies of shorebird species, few have observed sex-related adult survival (Larson *et al.* 2000, Sandercock *et al.* 2005, Stenzel *et al.* 2011), in contrast to other bird groups, in which females often have lower survival (e.g. Promislow *et al.* 1992, Grüebler *et al.* 2008). Sex-related adult survival may be rare amongst shorebirds partly because incubation is generally shared, compared with female-only incubation in other groups. Indeed, the male-biased ASR of many threatened bird species is thought to be largely attributed to the predation of incubating females (Donald 2007).

There are several possible reasons why we did not detect sex-related adult survival despite the fact that male Plovers incubated during the active period of the majority of predator species. First, if anti-predator behaviours are efficient even during the active period of predator species, predation may have little influence on the survival of either sex. Early flushing from the nest is the Plover's main response to potential predators of adults. Secondly, as survival is influenced by many aspects of the environment it could be diffcult to detect the influence of predation. Indeed, if the effects of other environmental variables on adult survival are also sex-related, they may balance out any in_uence of nocturnal predation on male survival. Finally, this is a rare and endangered species, and thus sample sizes are moderate and may lack sufficient power to detect small effect sizes.

Although predation from introduced species may be a conservation issue for St Helena Plovers, it appears unlikely that sex-related adult survival is exacerbating this. The absence of sex-related survival suggests that St Helena Plovers may have retained effective anti-predator behaviour from their ancestral forms that may have encountered nocturnal predators, despite the absence of native nocturnal predators on St Helena. One of the extinct rails endemic to St Helena may, however, have been an occasional egg-eater (Olson 1975). Finally, knowledge of sex-related demography also informs our understanding of the species' population dynamics and will advise conservation management.

Acknowledgements

Funding came from a University of Bath studentship, the RSPB and Chester Zoo. We would like to thank Annalea Beard, Robbie Watt and Chris Dodd of the St Helena National Trust, Eddie Duff, Will Kirby, the South Atlantic Invasive Species Project (an EU-funded RSPB initiative), the Government of St Helena and all those in St Helena who have supported this project. We thank Morten Frederiksen, Ruedi Nager and two anonymous reviewers for providing useful comments on previous drafts of this paper.

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